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The Isolation and Amino Acid Sequence of an Adrenocorticotrophin from the Pars Distalis and a Corticotrophin-Like Intermediate-Lobe Peptide from the Neurointermediate Lobe of the Pituitary of the Dogfish *Squalus acanthias*

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An adrenocorticotrophic hormone (ACTH) was isolated from extracts of the pars distalis of the pituitary of the dogfish *Squalus acanthias* by gel filtration and ion-exchange chromatography. It had 15% of the potency of human ACTH in promoting corticosteroidogenesis in isolated rat adrenal cells. Sequence analysis revealed it to be a nonatriacontapeptide with the following primary structure: Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Met-Gly-Arg-Lys-Arg-Arg-Pro-Ile-Lys-Val-Tyr-Pro-Asn-Ser-Phe-Glu-Asp-Glu-Ser-Val-Glu-Asn-Met-Gly-Pro-Glu-Leu. The *N*-terminal tridecapeptide sequence was identical with the proposed structure of dogfish α-melanocyte-stimulating hormone (α-MSH). On comparison with human ACTH eleven amino acid differences were seen, nine of which are in the 20–39 region of the molecule which is not essential for the steroidogenic activity of ACTH. A peptide identical with the 18–39 portion of this new ACTH was similarly isolated from the neurointermediate lobe of the pituitary where considerable amounts of dogfish α-MSH were found. This supported our view that ACTH as well as having a distinct biological role of its own is also the precursor of α-MSH.

The amino acid sequences of human, pig, ox and sheep adrenocorticotrophins are known and recently the structures of all these hormones have been revised or confirmed: human by Riniker *et al.* (1972) and Bennett *et al.* (1973); pig, Graf *et al.* (1971) and Riniker *et al.* (1972); and ox and sheep, Li (1972).

Biological activity corresponding to ACTH† has been detected in extracts of pituitaries from several lower vertebrate species: salmon (Rinfret & Hane, 1955; Fontaine-Bertrand *et al.*, 1969), cod (Woodhead, 1960) and elasmobranchs (DeRoos & DeRoos, 1967). Little is known of the molecular characteristics of these ACTH species and related peptides although recently Scott *et al.* (1971), using antibodies directed towards various parts of the mammalian ACTH molecule, have found cross-reaction in extracts of pituitaries of several vertebrate species. The lowest vertebrate studied in which both biological and immunological activity were present was the dogfish.

Although there was cross-reaction of dogfish pituitary extracts with an antiserum directed towards the 13–18 region of human ACTH, the displacement curve was not parallel to those given by standard human ACTH. As the primary structure of dogfish α-MSH is already known in this species (Lowry & Chadwick, 1970*a*), it was decided to determine the structure and properties of ACTH-like peptides in the dogfish, *Squalus acanthias*, with the possible further elucidation of the interrelationships of α-MSH, ACTH and, hopefully, the recently identified corticotrophin-like intermediate-lobe peptide which has been found in several mammalian species (residues 18–39 of ACTH; Scott *et al.*, 1973, 1974*a*,*b*). Since dogfish have a very distinct anatomical separation of the lobes of the pituitary responsible for ACTH production (the rostral end of the pars distalis) and MSH production (the neurointermediate lobe), this species was attractive for this investigation.

Materials and Methods

Materials

Microgranular CM-cellulose (CM-32) and DEAE-cellulose (DE-52) were obtained from Whatman Biochemicals Ltd. (Maidstone, Kent, U.K.) and Bio-Gel P-2 and P-6 (both 200–400 mesh) from Bio-Rad Laboratories (Richmond, Calif., U.S.A.).

Carboxypeptidase A, carboxypeptidase B (both treated with di-isopropyl phosphorofluoridate) and type XI trypsin (treated with diphenyl carbamoyl

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† Abbreviations: ACTH, adrenocorticotrophin; α-MSH, α-melanocyte-stimulating hormone.

chloride) were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Chymotrypsin (3× crystallized) was obtained from Worthington Enzyme Corp., Freehold, N.J., U.S.A. Aminopeptidase M was purchased from Rohm and Haas G.m.b.H. (Darmstadt, Germany) and treated with di-isopropyl phosphorofluoridate before use as described by Hill & Smith (1957). Cyanogen bromide was obtained from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.).

Trimethylamine was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Disposable polystyrene tubes (3ml capacity) were obtained from C. E. Payne and Sons Ltd., London S.W.4, U.K. and (15ml capacity) from Henleys Medical Supplies Ltd., London N8 0DL, U.K.

Pyridine, butyl acetate, phenyl isothiocyanate and trifluoroacetic acid were purified by the methods described by Lowry & Chadwick (1970*a*) and trimethylamine was purified as described by Bennett *et al.* (1973).

Edman-degradation steps were performed in 8ml screw-cap glass test tubes (Sovivel, France) treated with Repelcote water repellent (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.).

Synthetic mammalian α-MSH and synthetic human ACTH (revised sequence Sieber *et al.*, 1972) were kindly supplied by Dr. W. Rittel, CIBA–GEIGY Ltd. (Basle, Switzerland).

Methods

Biological and immunological assays. The isolated adrenal-cell method of Sayers *et al.* (1971), as modified by Lowry *et al.* (1973), was used to detect ACTH activity in column effluents and to estimate the potency of preparations, with synthetic human ACTH as standard.

The frog skin assay *in vitro* (Chadwick & Lowry, 1970) was used to estimate melanocyte-stimulating activity.

No assay was available for dogfish corticotrophin-like intermediate-lobe peptide, i.e. it has no known biological activity and none of the antibodies used in the detection of mammalian intermediate-lobe peptide (Scott *et al.*, 1974*a*,*b*) cross-reacted with extracts of dogfish neurointermediate lobes. Therefore pig peptide (30μg) (Scott *et al.*, 1974*b*) was added to crude dogfish material to act as an internal marker to help in the isolation and purification of dogfish corticotrophin-like intermediate-lobe peptide. This amount was equivalent to less than 1% of the final yield and therefore did not interfere with subsequent amino acid analyses and sequence analysis.

Extraction and isolation of pituitary peptides. The rostral region of the pars distalis and neurointermediate lobes of approx. 4000 dogfish were dissected separately from heads supplied by A. R. Jenner, Grimsby, Lincs., U.K., and collected in acetone at 4°C. The acetone was decanted and replaced daily for a period of 1 week and the resulting defatted tissue was extracted twice with acetic acid (10ml/1000 rostral lobes; 50ml/1000 neurointermediate lobes). After centrifugation at 4200*g* at 4°C the supernatants were freeze-dried. Each extract was then fractionated separately on the Bio-Gel P-2/P-6 system.

Gel-filtration and ion-exchange chromatography. Bio-Gel P-2 and P-6 and CM-cellulose columns were packed and run as described previously (Lowry & Chadwick, 1970*a*; Bennett *et al.*, 1973). The Bio-Gel P-2 and P-6 columns were in series so that the desalted effluent from the Bio-Gel P-2 column (2.5cm×2.5cm) ran directly on to the Bio-Gel P-6 column (2.5cm×120cm). DEAE-cellulose was fractionated in 0.5M-sodium acetate buffer, pH5.5, in a similar manner to that described by Hamilton (1958) and was packed and treated in the same way as the CM-cellulose. Peptides were detected automatically by an LKB Uvicord II or manually by a Unicam SP.500 spectrophotometer. Peptides that did not absorb u.v. light were detected by concentrating small amounts of each fraction, applying to silica t.l.c. plates and staining first with ninhydrin spray (BDH Chemicals Ltd.) and then with the chlorine method as described by Rydon & Smith (1952).

Disposable polystyrene test tubes were used throughout to decrease adsorption losses in the collection and handling of peptides. When minute quantities of material were present it was possible, because of the high volatility of the buffers used, to dry in the liquid states at 1330Pa (10mmHg) over P_2O_5 and NaOH.

Elution of ion-exchange columns. Ion-exchange columns were eluted with concave gradients formed by trimethylamine acetate buffer running from low to high molarity. This was achieved by using suitable beakers and conical flasks to contain the low- and high-molarity buffers respectively. A typical gradient would be formed by using a 20ml beaker as the mixing chamber and having the final buffer contained in a 25ml conical flask, the two being connected by a syphon. The total volume of the gradients mentioned indicate to what extent the system should be scaled up or down.

Purification of ACTH. The fractions with the bulk of ACTH activity obtained from the rostral-lobe extract after gel filtration on the Bio-Gel P-2/P-6 system were combined and freeze-dried and submitted to ion-exchange chromatography on CM-cellulose. The fractions after this purification which were thought to contain ACTH that had been oxidized during isolation were combined, freeze-dried, incubated at 37°C overnight in 0.5ml of thioglycollic acid and then repurified on CM-cellulose.

Purification of corticotrophin-like intermediate-lobe

peptide. Crude dogfish peptide was isolated from neurointermediate-lobe extracts from material which had $K_{av.}$ between 0.39 and 0.54 on the Bio-Gel P-2/P-6 system. ($K_{av.} = V_e - V_0/V_t - V_0$, where V_e is the elution volume, V_0 the void volume and V_t the total volume of the column.) The fractions involved are indicated on Fig. 1 of Bennett *et al.* (1974). After the addition of 30μg of pig peptide the mixture was rechromatographed on the Bio-Gel P-2/P-6 system. Material was located by use of an antiserum directed towards the 33–39 region of human ACTH (Ratcliffe *et al.*, 1972), which detected the pig peptide (Scott *et al.*, 1974*b*) added as marker to the crude dogfish material. Immunoreactive fractions were combined and freeze-dried and the dogfish peptide was finally purified on DEAE-cellulose.

Separation of peptide fragments. Tryptic and cyanogen bromide-cleaved fragments of dogfish ACTH and cyanogen bromide-cleaved fragments of dogfish intermediate-lobe peptide were separated on columns (0.4cm internal diam.×12cm length) of CM-cellulose. These were eluted by concave gradients running from 10mM- to 0.5M-trimethylamine acetate, pH5 (total vol. approx. 50ml), and 0.5ml fractions were collected every 20min.

Tryptic and peptic fragments of dogfish intermediate-lobe peptide and peptic fragments of *C*-terminal tryptic fragments of both dogfish ACTH and intermediate-lobe peptide were separated on columns (0.4cm internal diam.×12cm length) of DEAE-cellulose. These were developed by concave gradients running from 10mM- to 0.3M-trimethylamine acetate, pH5 (total vol. approx. 50ml), and 0.5ml fractions were collected every 20min.

Peptide fragmentations. Tryptic digestion of the ACTH (800μg, 165nmol) was carried out in 0.2ml of 0.05M-NH_4HCO_3 buffer (pH8.5) with a peptide/enzyme ratio of 150:1 (w/w) for 3h at 37°C. The resulting peptide fragments were subjected to ion-exchange chromatography on CM-cellulose and small amounts analysed for amino acid content. All fractions containing peptide fragments were combined and the solution was freeze-dried and treated with trypsin again [conditions as above except peptide/enzyme ratio, 50:1 (w/w) for 4h]. On further chromatography this gave more fragments which aided sequence analysis.

Tryptic hydrolysis of the intermediate-lobe peptide was carried out under the latter conditions and fragments were separated by ion-exchange chromatography on DEAE-cellulose.

Pepsin digestion was carried out in 0.2ml of 0.01M-HCl with a peptide/enzyme ratio of 150:1 (w/w) for 4h at 37°C and fragments were separated by ion-exchange chromatography on DEAE-cellulose.

Cyanogen bromide cleavage at methionine residues was carried out by using the method of Steers *et al.* (1956) on approx. 200μg of peptide and separation of fragments was achieved by ion-exchange chromatography on CM-cellulose.

Amino acid analyses. These were performed by using either a Beckman 120 C or a Technicon AutoAnalyzer modified to give increased sensitivity as described by Bennett *et al.* (1973). Peptide samples were hydrolysed either in 0.2ml of 6M-HCl in evacuated glass tubes for 16h at 110°C (with a crystal of phenol to prevent tyrosine loss when appropriate) or by complete enzymic digestion with Sepharose-bound peptidases as described by Bennett *et al.* (1972).

Glutamine and asparagine were determined by using the Beckman 120C amino acid analyser employing the lithium citrate buffer described by Benson *et al.* (1967).

Sequence analysis. *N*-Terminal sequence analysis of the whole peptide was carried out by using the subtractive Edman degradation of Gray (1967) as modified by Lowry & Chadwick (1970*a*). Degradation steps were performed in 8ml screw-cap test tubes. Aminopeptidase M digestions were performed in 0.03M-sodium phosphate buffer (pH7.5) at 37°C with a peptide/enzyme ratio of 200:1 (w/w) for various times.

C-Terminal analysis was carried out by using carboxypeptidases A and B in 0.05M-Tris buffer containing 0.2M-NaCl (pH8.5) at 25°C with a peptide/enzyme ratio of 50:1 (w/w) for carboxypeptidase A and 100:1 (w/w) for carboxypeptidase B for various times.

Release of amino acids by these enzymes was estimated, after quenching the incubations with 0.1M-HCl, by analysis for free amino acids in the supernatants.

Results

Figs. 1 and 2 show the results from the different steps involved in the purification of dogfish ACTH

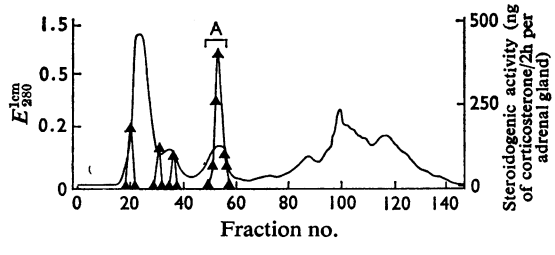

Fig. 1. *Bio-Gel P-2 and P-6 gel-filtration chromatography of a freeze-dried acetic acid extract* (210*mg*) *of approx.* 4000 *dogfish rostral lobes*

——, E_{280}; ▲, steroidogenic activity. The column (2.5cm internal diam.×25cm length of Bio-Gel P-2 and that (2.5cm internal diam.×120cm length) of Bio-Gel P-6 were eluted with 1M-acetic acid and 5ml fractions were collected every 20min.

by column chromatography. The large peak of ACTH biological activity (A) (72% of original extract) on the Bio-Gel P-2/P-6 system after freeze-drying, ran as a reasonably homogeneous peak on CM-cellulose (Fig. 2). The biologically active fraction F_2 (53% of original extract) (Fig. 2) was freeze-dried and samples were taken for acid and enzyme hydrolysis (see Table 1). The molar ratios indicate that the peptide isolated was homogeneous and contained 39 amino acid residues. Because of the high methionine content and integral molar ratios of amino acids for fraction F_2, fraction F_1 (Fig. 2) was assumed (cf. Dedman *et al.*, 1961) to contain methionine sulphoxide analogues of dogfish ACTH formed during isolation, and fraction F_1, after treatment with thioglycollic acid and repurification on CM-cellulose, yielded homogeneous material which was biologically active and gave similar amino acid analyses to those shown in Table 1. The total yield of dogfish ACTH from the 4000 pituitaries used was 1.8mg and the biological potency, as compared with synthetic human ACTH in the isolated rat adrenal cell assay, was 0.15 with 95% confidence limits of 0.11–0.21. It was approximately equivalent to human ACTH in the MSH bioassay.

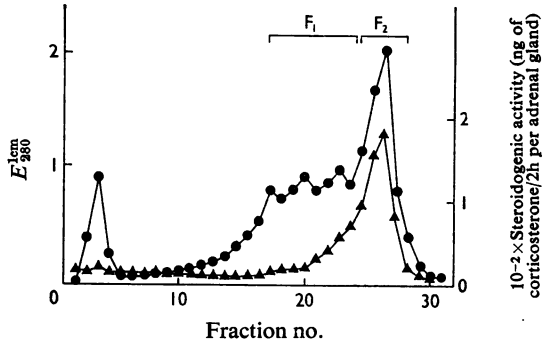

Fig. 2. *CM-cellulose ion-exchange chromatography of ACTH-active material from approx.* 4000 *dogfish rostral lobes after purification on Bio-Gel P-2 and P-6*

●, E_{280}; ▲, steroidogenic activity. The column (0.6cm internal diam. × 20cm length) was developed by a concave gradient running from 0.3M- to 0.5M-trimethylamine acetate, pH5 (total vol. 50ml), and 1ml fractions were collected every 20min. Fraction F_2 was biologically active. Fraction F_1 was treated with thioglycollic acid and rechromatographed in a similar manner to produce more biologically active ACTH.

The crude fraction of dogfish intermediate-lobe peptide (marked in Fig. 1 of Bennett *et al.*, 1974) was rechromatographed with pig peptide as an internal marker on the Bio-Gel P-2/P-6 system. Fractions containing pig peptide were assumed to contain dogfish peptide and after freeze-drying were submitted to ion-exchange chromatography on DEAE-cellulose. The bulk of retarded u.v.-absorbing material ran as a separate symmetrical peak (Fig. 3) and on this basis the peptide responsible was tentatively called dogfish corticotrophin-like intermediate-lobe peptide. The amino acid analyses of a sample of this peptide are shown in Table 1. The yield from 2000 neuro-intermediate lobes was 7.2mg. There were insignificant amounts of ACTH biological activity in the eluate of the Bio-Gel P-2/P-6 fractionation of the neurointermediate-lobe extract.

If the amino acid analysis of the presumed dogfish intermediate-lobe peptide is subtracted from that of dogfish ACTH, then the resulting analysis apart from two simple substitutions (methionine for valine and arginine for lysine) is identical with the amino acid content of the 1–17 sequence of mammalian ACTH.

Separate digestions of dogfish ACTH with aminopeptidase M and carboxypeptidase A revealed serine to be at the *N*-terminus and leucine to be at the *C*-terminus.

Similar treatment of dogfish corticotrophin-like intermediate peptide with carboxypeptidase A yielded leucine as the only free amino acid in the incubation mixture. Arginine was at the *N*-terminus of dogfish peptide, as the amino acid analysis after one Edman degradation step was the same as the original peptide

Table 1. *Amino acid analyses (molar ratios) of dogfish ACTH and corticotrophin-like intermediate-lobe peptide after acid hydrolysis and Sepharose-bound enzyme hydrolysis*

For details see the text.

Amino acid ...	Asp	Asn	Ser	Glu*	Pro	Gly	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp
ACTH, acid hydrolysis	3.18	—	3.39	5.03	4.16	2.93	2.00	2.93	0.96	1.00	2.02	2.11	3.00	1.05	4.05	0.69
ACTH, enzyme hydrolysis	1.26	1.79	3.85	4.76	4.00	2.93	2.00	2.73	1.04	0.90	1.99	2.03	3.06	1.06	3.95	0.95
Intermediate-lobe peptide acid hydrolysis	3.08	—	1.81	4.03	3.01	1.03	1.96	0.91	0.96	1.03	0.98	1.03	0.90	—	1.00	—
Intermediate-lobe peptide, enzyme hydrolysis	1.20	1.84	1.99	3.99	3.14	1.03	1.99	0.98	1.10	1.05	0.99	1.01	0.83	—	1.03	—

* No glutamine was found after enzyme hydrolysis.

apart from the disappearance of 83% of the arginine. The enzyme digestion results are summarized in Table 2.

The amino acid molar ratios and sequence analysis of dogfish ACTH tryptic peptides which had previously been separated on CM-cellulose are shown in Table 3 and summarized in Fig. 4.

As leucine was the *C*-terminal amino acid, peptide AT_7 must be the *C*-terminal octapeptide and as serine was the *N*-terminal amino acid, peptide AT_1 must be the *N*-terminal octapeptide of the ACTH. In addition the former peptide does not contain arginine or lysine. Peptide AT_6 formed by mild tryptic hydrolysis of dogfish ACTH has exactly the same amino acid analysis as that proposed for dogfish intermediate-lobe peptide (see Table 1 and Fig. 4), and as there is only one isoleucine residue in the whole molecule, this assigns peptides AT_3, AT_4 and AT_5 to positions 16–21, 17–21 and 18–21 respectively and in addition implies the sequence of residues 16–17 to be Lys-Arg. This is confirmed by the sequencing data of peptides AT_3 and AT_4. From the assignments of peptides AT_1, AT_3 and AT_7, peptide AT_2 must represent residues 9–15 (Fig. 4). This is corroborated by the amino acid analysis of cyanogen bromide-cleaved fragments shown in Table 4 and Fig. 4.

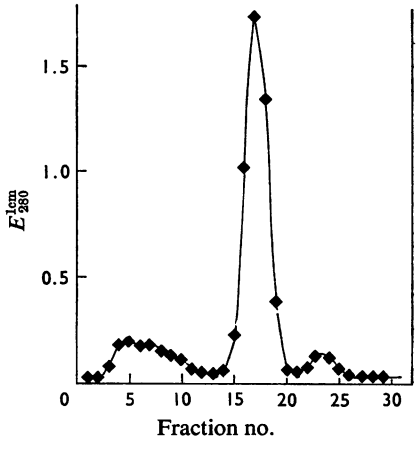

Fig. 3. *DEAE-cellulose ion-exchange chromatography of material from about* 1000 *neurointermediate lobes which had the same chromatographic properties as pig ACTH* 18–39 *on the Bio-Gel P-2/P-6 system*

The column (0.4cm internal diam. × 15cm length) was developed by a concave gradient running from 0.1M- to 0.3M-trimethylamine acetate, pH5 (total vol. 50ml), and 0.5ml fractions were collected every 20min.

The tryptic fragments of dogfish intermediate-lobe peptide (Table 3), which had been separated on DEAE-cellulose, peptides CT_1 (18–21) and CT_2 (22–39), gave identical analyses with their appropriate counterparts in the ACTH molecule (peptides AT_5 and AT_7), and in addition peptide CT_1 had the same sequence as peptide AT_5, i.e. Arg-Pro-Ile-Lys (Fig. 5). The positioning of the tryptophan residue in peptide AT_2 (9–15) was determined by aminopeptidase M digestion (Table 2). Prolonged treatment with this enzyme released only tryptophan and glycine in equimolar amounts plus a resistant peptide AT_{2APM}, and when this mixture was subjected to the subtractive Edman-degradation procedure lysine disappeared from the analysis of the residual peptide as well as glycine and tryptophan (Table 3). Proline was the next amino acid in the sequence and the tertiary amide bond between $Lys_{(11)}$ and $Pro_{(12)}$ explains the resistance of this bond to both aminopeptidase and trypsin action. The sequence analysis of the tryptic fragments of dogfish ACTH presented in Table 3 leads to the assignment of the sequence of residues 1–21 as: Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Met-Arg-Lys-Arg-Arg-Pro-Ile-Lys.

Peptides AT_7 and CT_2 were resistant to chymotrypsin digestion even after 18h at 37°C with a peptide/enzyme ratio of 50:1 (w/w). Both peptides, however, were susceptible to digestion with pepsin and after separation of the products on DEAE-cellulose yielded peptides with very similar analyses, i.e. $AP_1 = CP_4$, $AP_3 = CP_5$ (Table 5). The peptide equivalent to peptide AP_2 in the peptic digest of peptide CT_2 was lost in the initial separation but was, however, obtained from pepsin-treated intermediate-lobe peptide itself (peptide CP_2, Table 5).

Table 2. *Amino acids released from dogfish ACTH and the intermediate-lobe peptide and three ACTH tryptic fragments by digestion with carboxypeptidases A and B and aminopeptidase M*

Times of incubation are shown in parentheses. For other details see the text.

	Amino acids released and incubation times				
	ACTH	Intermediate-lobe peptide	Peptide AT_1 (1–8)	Peptide AT_2 (9–15)	Peptide AT_5 (18–21)
Carboxypeptidase A	Leu (4h)	Leu (4h)	—	—	—
Carboxypeptidase B	—	—	Arg > Phe (¼h)	Arg (1h)	Lys (1h)
Aminopeptidase M	Ser (5h)	—	—	Trp > Gly (½h)	—

Table 3. *Amino acid compositions (molar ratios) of the tryptic fragments of dogfish ACTH and corticotrophin-like intermediate-lobe peptide and of the residual peptides resulting from stepwise subtractive Edman degradation (for ACTH the peptides are prefixed A and for intermediate-lobe peptide they are prefixed C)*

For experimental details see the text. At each step an *N*-terminal residue is removed and the identification of the amino acid deleted between each successive step (indicated by *) therefore gives the sequence of the peptide. The first two residues of peptide AT_2 were released by incubation with aminopeptidase M for 1 h and the residual peptide was called peptide AT_{2APM} (for other conditions see the text and Table 2). The mixture was then subjected to the subtractive Edman procedure whereupon the free amino acids as well as the *N*-terminal amino acid of the residual peptide were removed at the first step. For peptide identification see Figs. 4 and 5. Order of evolution of the tryptic peptides of ACTH from the CM-cellulose column and recoveries from 160nmol of starting material were: (1) AT_7, 110nmol; (2) AT_6, 30nmol; (3) AT_1, 125nmol; (4) AT_5, 25nmol; (5) AT_2, 110nmol; (6) AT_4, 52nmol; (7) AT_3, 18nmol. Order of elution and recoveries of tryptic peptides on the DEAE-cellulose column from 200nmol of the intermediate-lobe peptide were: (1) CT_1, 172nmol; (2) CT_2, 165nmol.

Peptide fragment / Amino acid ...	Asx	Ser	Glu	Pro	Gly	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp
AT_1 (1–8)	—	1.5	1.2	—	—	—	0.8	—	—	0.9	1.0	—	0.9	1.1	—
1st step	—	0.9*	1.2	—	—	—	0.9	—	—	0.9	1.0	—	1.1	1.0	—
2nd step	—	0.9	1.2	—	—	—	0.9	—	—	0.1*	1.0	—	1.0	1.0	—
3rd step	—	0.2*	1.1	—	—	—	0.8	—	—	0.1	1.0	—	1.0	1.1	—
4th step	—	0.2	1.1	—	—	—	0.1*	—	—	0.0	0.8	—	1.1	0.9	—
†AT_2 (9–15)	—	—	—	1.1	1.9	—	1.0	—	—	—	—	1.0	—	1.2	0.8
AT_{2APM} (11–15)															
1st step	—	—	—	0.9	1.2	—	0.9	—	—	—	—	0.2*	—	1.0	—
2nd step	—	—	—	0.2*	0.9	—	1.1	—	—	—	—	0.1	—	1.1	—
3rd step	—	—	—	0.0	0.9	—	0.2*	—	—	—	—	0.0	—	1.1	—
4th step	—	—	—	0.0	0.3*	—	0.0	—	—	—	—	0.0	—	1.0	—
AT_3 (16–21)	—	—	—	1.0	—	—	—	1.1	—	—	—	2.0	—	2.0	—
1st step	—	—	—	1.3	—	—	—	0.9	—	—	—	0.6*	—	2.2	—
AT_4 (17–21)	—	—	—	1.0	—	—	—	1.0	—	—	—	1.1	—	2.3	—
1st step	—	—	—	1.1	—	—	—	0.9	—	—	—	0.9	—	1.1*	—
2nd step	—	—	—	1.1	—	—	—	1.0	—	—	—	0.9	—	0.3*	—
3rd step	—	—	—	0.2*	—	—	—	1.1	—	—	—	0.9	—	0.3	—
4th step	—	—	—	0.0	—	—	—	0.3*	—	—	—	1.0	—	0.3	—
AT_5 (18–21)	—	—	—	1.0	—	—	—	1.0	—	—	—	1.0	—	1.0	—
AT_6 (18–39)	3.1	2.0	4.0	2.8	1.3	2.0	0.9	1.1	1.1	1.1	1.1	1.0	—	1.0	—
AT_7 (22–39)	3.1	1.6	4.4	1.5	1.2	2.0	0.9	—	1.3	1.0	1.1	—	—	—	—
1st step	2.7	1.8	3.7	1.0	1.3	1.0*	0.7	—	1.0	1.0	1.0	—	—	—	—
CT_1 (18–21)	—	—	—	1.0	—	—	—	1.0	—	—	—	1.0	—	1.0	—
1st step	—	—	—	1.0	—	—	—	1.0	—	—	—	1.0	—	0.2*	—
2nd step	—	—	—	0.1*	—	—	—	0.9	—	—	—	1.1	—	0.2	—
3rd step	—	—	—	0.0	—	—	—	0.0*	—	—	—	1.0	—	0.0	—
CT_2 (22–39)	2.9	1.8	3.9	2.1	1.0	1.9	0.9	—	1.0	1.0	1.0	—	—	—	—

† Hydrolysis by Sepharose-bound enzymes (all others are by acid hydrolysis).

Exploratory sequence results on the peptide pairs AP_1, CP_4 and AP_3, CP_5 provided further evidence for their similarity and to avoid unnecessary additional sequencing the appropriate peptides were combined and sequenced together. At every stage during the sequential degradation these mixtures behaved as homogeneous peptides. The sequence analysis of peptides AP_2 and AC_4 gave similar results to those of the corresponding peptides from the intermediate lobe (CP_2, CC_2) (Tables 4 and 5).

Two asparagine residues were present in each of the ACTH and intermediate-lobe peptide molecules (Table 1) and these occurred singly in each of the peptides AP_1, CP_4 and AP_3, CP_5. As there are no aspartic acid residues in any of these peptides there was no difficulty in assigning the positions of the two amide-containing amino acids.

As leucine occurs only once at the *C*-termini of both molecules, both AP_3 and CP_3 are the *C*-terminal peptides, i.e. 31–39 [the *C*-terminal tetrapeptide

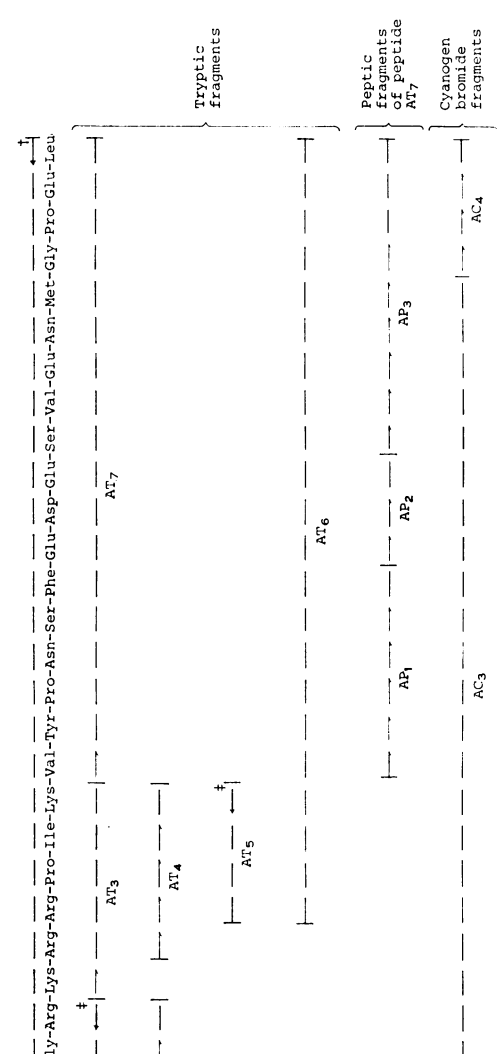

Fig. 4. *Summary of the sequence analysis of dogfish ACTH*

→, Subtractive Edman-degradation steps; → or ←, exopeptidase digestion resulting from treatment with aminopeptidase M (*), carboxypeptidase A (†) or carboxypeptidase B (‡).

Fig. 5. *Summary of the sequence analysis of dogfish corticotrophin-like intermediate-lobe peptide*

→, Subtractive Edman-degradation steps; ←, exopeptidase digestion resulting from treatment with carboxypeptidase A.

Table 4. *Amino acid compositions (molar ratios) of the cyanogen bromide-cleaved fragments of dogfish ACTH, its tryptic fragment AT_1, dogfish intermediate-lobe peptide and the residual peptides resulting from their stepwise subtractive Edman degradation (for ACTH the peptides are prefixed A and for intermediate-lobe peptide they are prefixed C)*

For experimental details see the text. At each step an *N*-terminal residue is removed and the identification of the amino acid depleted between each successive step (indicated by *) therefore gives the sequence of the peptide. The presence of homoserine (Hse) is indicated by +. For peptide identification see Figs. 4 and 5. All cyanogen bromide-cleaved fragments were separated on CM-cellulose. Recoveries and order of elution of fragments from 44nmol of ACTH were: (1) AC_4, 22nmol; (2) AC_1, 28nmol; (3) AC_3, 21nmol; (4) AC_2, 12nmol. Similar treatment of 146nmol of the intermediate-lobe peptide gave: (1) CC_2, 110nmol; (2) CC_1, 102nmol; and of peptide AT_1 (40nmol) gave: (1) AT_1C_1, 18nmol; (2) AT_1C_2, 25nmol.

Amino acid ... Asx Peptide fragment	Asx	Ser	Hse	Glu	Pro	Gly	Val	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp
AC_1 (1–4)	—	2.0	+	—	—	—	—	—	—	1.1	—	—	—	—	—
AC_2 (5–13)	—	—	+	1.1	1.1	1.1	—	—	—	—	0.8	1.3	1.2	1.1	0.7
AC_3 (14–35)	2.8	1.6	+	3.0	1.8	1.3	2.1	1.2	—	1.2	1.1	2.1	—	3.5	—
AC_4 (36–39)	—	—	—	1.0	0.9	1.1	—	—	1.0	—	—	—	—	—	—
1st step	—	—	—	1.0	0.9	0.1*	—	—	0.9	—	—	—	—	—	—
2nd step	—	—	—	1.2	0.1*	0.1	—	—	0.8	—	—	—	—	—	—
3rd step	—	—	—	0.0*	0.0	0.0	—	—	1.0	—	—	—	—	—	—
AT_1C_1 (1–4)	—	2.0	+	—	—	—	—	—	—	1.0	—	—	—	—	—
AT_1C_2 (5–8)	—	—	—	1.2	—	—	—	—	—	—	0.9	—	0.9	1.1	—
1st step	—	—	—	0.4*	—	—	—	—	—	—	1.1	—	1.1	0.9	—
2nd step	—	—	—	0.4	—	—	—	—	—	—	0.9	—	0.5*	1.1	—
CC_1 (18–35)	3.1	1.8	+	3.1	2.3	—	2.1	1.0	—	1.1	1.2	1.0	—	1.2	—
CC_2 (36–39)	—	—	—	1.0	1.2	1.0	—	—	1.0	—	—	—	—	—	—
1st step	—	—	—	1.0	1.1	0.1*	—	—	0.9	—	—	—	—	—	—
2nd step	—	—	—	1.1	0.0*	0.0	—	—	0.9	—	—	—	—	—	—
3rd step	—	—	—	0.3*	0.0	0.0	—	—	1.0	—	—	—	—	—	—

† Hydrolysis by Sepharose-bound enzymes (all others are acid hydrolysis).

of both these peptides was sequenced by using cyanogen bromide-cleaved fragments AC_4 and CC_2 (Table 4)].

Since valine was found at the *N*-terminus of peptide AT_7 (Table 3) and all the amino acids of peptide CP_6, except glutamic acid, were found in peptide CP_1 (formed by pepsin digestion of the whole intermediate-lobe peptide molecule, Table 4), peptides AP_1 and CP_1 must occur at positions 22–27 and 18–27 respectively. The position of the glutamic acid in peptide CP_6 at position 28 is inferred. The Glu-Asp-Glu tripeptide sequence of both peptides AP_2 and CP_2 can only be assigned to positions 28–30 in both molecules [the Asp residue in this peptide was also proved by enzymic digestion, although a very prolonged hydrolysis time (47h) was needed to digest this very resistant sequence].

The common sequence of residues 22–39 of both dogfish ACTH and corticotrophin-like intermediate-lobe peptide is therefore: Val-Tyr-Pro-Asn-Ser-Phe-Glu-Asp-Glu-Ser-Val-Glu-Asn-Met-Gly-Pro-Glu-Leu.

The complete sequence of both peptides is shown in Figs. 4 and 5.

Discussion

Although chondrichthyeans (cartilaginous fish) are the lowest vertebrate group in which there is firm evidence for a pituitary–interrenal axis (see DeRoos & DeRoos, 1967), the primary structure of dogfish ACTH shows a striking resemblance in both the sequence and number of amino acid residues to the known mammalian adrenocorticotrophins (Fig. 6). In the so-called steroidogenic region, i.e. residues 1–19, there are only two very conservative substitutions, methionine for valine at residue 13 and arginine for lysine at residue 15. Both of these changes only represent single base changes in the genetic code. As dogfish have been separated from the main line of vertebrate evolution for more than 300 million years this suggests that this portion of the molecule had already acquired a biological role early in vertebrate evolution and subsequently has been under very strong selection pressure. There are, however, nine amino acid residue substitutions in the 20–39 *C*-terminal region compared with human ACTH. Nearly half of these substitutions require two base changes in the genetic code although the

Table 5. *Amino acid compositions (molar ratios) of the peptic fragments of dogfish ACTH tryptic fragment AT_7 and the intermediate-lobe peptide and of the residual peptides resulting from their stepwise subtractive Edman degradation (for ACTH the peptides are prefixed A and for intermediate-lobe peptide they are prefixed C)*

For sequence analysis peptide AP_1 was combined with peptide CP_4 and peptide AP_3 with peptide CP_5. For experimental details see the text. At each step an *N*-terminal residue is removed and the identification of the amino acid depleted between each successive step (indicated by *) therefore gives the sequence of the peptide. For peptide identification see Figs. 4 and 5. All peptides were separated on the DEAE-cellulose column. Recoveries from a peptic digestion of 90nmol of peptide AT_7 and elution order were: (1) AP_1, 68nmol; (2) AP_3, 61nmol; (3) AP_2, 57nmol. Similarly a peptic digestion of peptide CT_2 gave: (1) CP_3, 80nmol; (2) CP_6, 6nmol; (3) CP_5, 62nmol; and a peptic digestion of 100nmol of the intermediate-lobe peptide gave: (1) CP_1, 82nmol; (2) CP_3, 62nmol; (3) CP_2, 48nmol.

	Amino acid ... / Peptide fragment	Asx	Ser	Glu	Pro	Gly	Val	Met	Ile	Leu	Tyr	Phe	Lys	Arg
	AP_1 (22–27)	1.0†	0.9	—	1.0	—	1.0	—	—	—	1.0	1.0	—	—
	CP_4 (22–27)	1.1†	1.0	—	0.9	—	1.0	—	—	—	1.0	0.8	—	—
Peptides AP_1+CP_4	1st step	1.0	0.9	—	1.0	—	0.0*	—	—	—	1.1	1.0	—	—
	2nd step	1.1	1.0	—	1.0	—	0.0	—	—	—	0.0*	1.0	—	—
	3rd step	1.1	0.9	—	0.1*	—	0.0	—	—	—	0.0	1.0	—	—
	4th step	0.1*	1.0	—	0.0	—	0.0	—	—	—	0.0	1.0	—	—
	5th step	0.0	0.3*	—	0.0	—	0.0	—	—	—	0.0	1.0	—	—
	AP_2 (28–30)	1.1‡	—	1.9	—	—	—	—	—	—	—	—	—	—
	1st step	1.0	—	1.3*	—	—	—	—	—	—	—	—	—	—
	2nd step	0.4*	—	1.0	—	—	—	—	—	—	—	—	—	—
	AP_3 (31–39)	1.1†	0.9	2.0	1.2	0.9	1.0	0.9	—	1.0	—	—	—	—
	CP_5 (31–39)	1.1†	0.9	2.2	1.0	0.9	1.0	0.8	—	1.1	—	—	—	—
Peptides AP_3+CP_5	1st step	1.1	0.1*	2.2	0.8	1.0	0.9	0.9	—	1.0	—	—	—	—
	2nd step	1.1	0.1	1.7	1.0	1.1	0.1*	0.9	—	1.1	—	—	—	—
	3rd step	1.0	0.0	1.1*	0.9	1.0	0.0	0.9	—	1.1	—	—	—	—
	4th step	0.2*	0.1	1.3	0.9	1.0	0.1	0.7	—	1.1	—	—	—	—
	5th step	0.1	0.1	1.2	0.8	0.9	0.0	0.1*	—	1.1	—	—	—	—
	6th step	0.0	0.1	1.1	0.9	0.4*	0.1	—	—	0.9	—	—	—	—
	CP_1 (18–27)	1.0	0.8	—	2.0	—	1.0	—	1.0	—	1.0	0.9	1.0	1.0
	CP_2 (28–30)	0.9‡	—	2.0	—	—	—	—	—	—	—	—	—	—
	1st step	0.9	—	1.1*	—	—	—	—	—	—	—	—	—	—
	2nd step	0.5*	—	1.0	—	—	—	—	—	—	—	—	—	—
	CP_3 (31–39)	1.0	0.9	1.8	1.1	1.0	1.0	0.7	—	1.2	—	—	—	—
	CP_6 (22–28)	1.0	1.0	1.0	1.0	—	1.0	—	—	—	1.1	1.1	—	—

† Amino acid analysis after Sepharose–enzyme hydrolysis revealed these to be asparagine residues.
‡ Amino acid analysis after Sepharose–enzyme hydrolysis revealed these to be aspartic acid residues.

general hydrophobic/hydrophilic properties of most of these positions remain unchanged. However, despite the high degree of substitution it can be seen that in the *C*-terminal region all the acidic residues are in exactly the same positions as in human ACTH. This remarkable feature in the so-called non-essential portion of ACTH suggests a possible function for these residues, either neutralizing the very basic part (8–21) of the molecule or taking part in some secondary structural formation, e.g. internal salt bridging.

The similarities between dogfish and mammalian ACTH species suggest that the overall mutation rate of ACTH has not been particularly high in the 300 million years since cartilaginous fish diverged from the main line of vertebrate evolution. This might be expected for small biologically active molecules where a large portion of the sequence partakes in binding at receptors, i.e. the *N*-terminal octadecapeptide sequence here is required for full biological activity (Maier *et al.*, 1971).

The low biological activity of the dogfish ACTH (15%) compared with human ACTH on rat adrenal cells is probably explained by the change in charge of the very basic region 15–18. The epsilon amino groups of lysine in such a region may often be

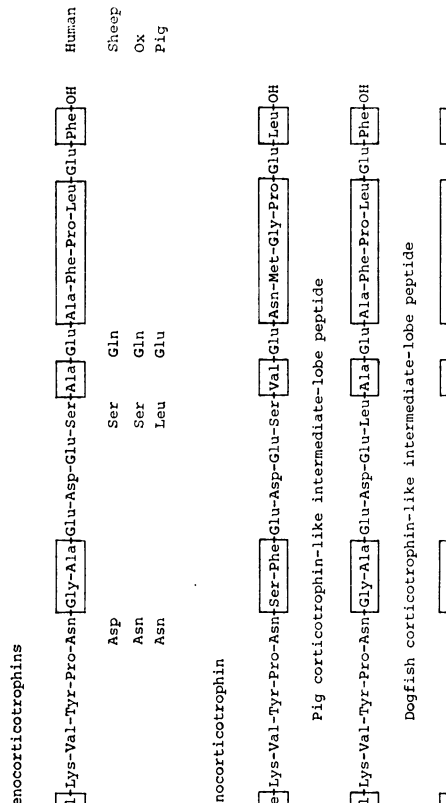

Fig. 6. *Amino acid sequences of mammalian and dogfish adrenocorticotrophins (α-MSH) and the corresponding intermediate-lobe peptides*

Solid lines enclosing loci indicate where differences occur.

only partly charged at physiological pH values; thus the change of Lys to Arg at position 15 would not only change the charge characteristic at this position but also that on the lysine at position 16. These changes could therefore result in a change in binding to adrenal receptors.

The asparagine residue at position 25 and the glutamic acid residue at position 33, which have been the subject of recent revision in the mammalian ACTH species (Riniker *et al.*, 1972; Li, 1972), are retained, although Li (1972) still finds an aspartic acid residue at position 25 in sheep ACTH and glutamine residues at position 33 in both the ox and sheep molecules. In the light of our information from a much more primitive animal it would appear that the ox and sheep hormones may warrant further examination at these positions.

During enzyme treatment of the *C*-terminal tryptic fragments of both the peptides, the bond $Phe_{(27)}$–$Glu_{(28)}$, although susceptible to pepsin, was completely resistant to hydrolysis by chymotrypsin under the conditions used. This is probably due to the proximity of the very acid region 28–30, which indeed required prolonged digestion with Sepharose-bound enzymes. Dogfish α-MSH (Lowry & Chadwick, 1970*a*; Bennett *et al.*, 1974) constitutes the *N*-terminal tridecapeptide sequence of dogfish ACTH, the methionine substitution at residue 13 being present in both molecules. Dogfish intermediate-lobe peptide isolated from the neurointermediate lobe was identical with the 18–39 region of dogfish ACTH. Corticotrophin-like intermediate-lobe peptide was also present in considerable quantities in the neurointermediate lobe (approx. 4 μg/lobe) which are similar to the amounts of dogfish α-MSH also occurring in this lobe (Lowry & Chadwick, 1970*a*). All these observations are completely analogous with those found for two species of mammals, the rat and the pig (Scott *et al.*, 1974*a*,*b*).

The rostral end of the pars distalis (where dogfish ACTH is found) and the neurointermediate lobe (where there is dogfish α-MSH, corticotrophin-like intermediate-lobe peptide and no detectable ACTH biological activity) are anatomically completely separated in this animal. This information lends considerable weight to the theory that not only do α-MSH and ACTH come from the same gene (Lowry & Chadwick, 1970*b*) but that ACTH is synthesized and acts as the precursor for α-MSH in the pars intermedia cells of the vertebrate pituitary, corticotrophin-like intermediate-lobe peptide being found as the *C*-terminal part of ACTH that is left (Scott *et al.*, 1973, 1974*a*,*b*). As the lamprey, an animal which diverged from the common line of evolution before the elasmobranchs, has a well-developed MSH–melanophore system without the presence of true inter-renal tissue, this suggests that ACTH may be the more primitive peptide, although

melanin dispersion may be the more primitive function.

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