A STUDY OF VERTEBRATE CORTICOTROPHINS AND RELATED PEPTIDES

Alexander P. Scott

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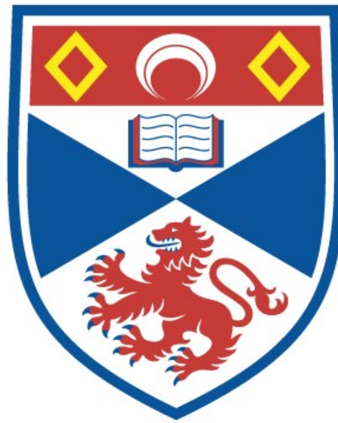

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UNIVERSITY OF ST. ANDREW'S, FIFE, SCOTLAND

A STUDY OF VERTEBRATE CORTICOTROPHINS AND RELATED PEPTIDES

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A thesis submitted to the University of
St. Andrew's for the degree of
Doctor of Philosophy

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I declare that this thesis has been composed by myself and the work of which it is a record has been done by myself. It has not been accepted in any previous application for a higher degree. The date of admission as a research student and candidate for a Ph.D., was October 1st, 1968.

....

A. P. Scott.

I declare that the conditions of the Ordinance and Regulations (Ph.D.) have been fulfilled.

....

Supervisor

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ABBREVIATIONS

ACTH - adrenocorticotrophic hormone

MSH - melanocyte stimulating hormone

LPH - lipotrophic hormone

CLIP - corticotrophin-like intermediate lobe peptide

Asp - aspartic acid (Asn - asparagine , Asx - either Asp or Asn)

Thr - threonine

Ser - serine

Glu - glutamic acid (Gln - glutamine , Glx - either Glu or Gln)

Pro - proline

Gly - glycine

Ala - alanine

Cys - cystine

Val - valine

Met - methionine

Leu - leucine

Ile - isoleucine

Tyr - tyrosine

Phe - phenylalanine

Lys - Lysine.

His - histidine

Arg - arginine

Try - tryptophan

HCl - hydrochloric acid

HAc - acetic acid

TFA - trifluoroacetic acid

Me_3NAc - trimethylamine acetate

I - iodine

g - gram

mg - milligram 10^{-3}g

μg - microgram 10^{-6}g

ng - nanogram 10^{-9}g

pg - picogram 10^{-12}g

M - molar

N - normal

mM - millimolar

nM - nanomolar

cm - centimetre

mm - millimetre 10^{-3}metre

nm - nanometre 10^{-9}metre

ml - millilitre 10^{-3}litre

μl - microlitre 10^{-6}litre

I.S - international standard

I.U - international units

mU - milliunits 10^{-3}U

μU - microunits 10^{-6}U

v/v - volume/volume

w/v - weight/volume

IV - intravenous

S.E.- standard error

S.D.- standard deviation

sec - second

min - minute

h - hour

% - percentage

° - temperature in degrees centigrade

rpm - revolutions per minute

cps - counts per second

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ABSTRACT

Literature concerning the structure and localisation of ACTH, MSH and related peptides in mammalian pituitaries has been reviewed and the evidence for ACTH-like peptides in non-mammalian vertebrates summarised.

The structure of ACTH in non-mammalian vertebrates was studied by bioassay and radioimmunoassay. Corticotrophin immunoreactivity in pituitary extracts from a variety of species was compared with that of natural human ACTH, using five antisera which cross-reacted with different parts of the ACTH molecule. The predominant binding sites of the antisera were determined by studies with synthetic ACTH fragments. Bioactivity and N-terminal immunoactivity were found in pituitary extracts of all species studies. Differences in reactivity with the N-terminally directed antisera indicated structural differences between corticotrophins of the lower vertebrates. Only reptilian, avian and mammalian pituitary extracts cross-reacted with C-terminally directed antisera.

Whole reptile and mammal pituitaries were found to contain more C- than N-terminal activity. Determination of the relative concentrations of these activities in rat and guinea-pig pituitary lobes showed that the posterior lobes contained a large excess of C-terminal activity, which was released into the media in organ culture. Fluorescent staining indicated that C-terminal activity was located in the pars intermedia. C-terminal immunoactive peptides were isolated from rat and pig pituitaries and a human tumour. On gel filtration they eluted between ACTH and α-MSH, and behaved as acidic peptides on ion-exchange chromatography. Amino acid and end group

analysis showed that they closely resembled the α^{18-39} portion of ACTH. Consequently they have been termed 'Corticotrophin-like Intermediate lobe Peptide', (CLIP).

The significance of the comparative studies with crude pituitary extracts of vertebrate origin and of the C-terminally immunoactive peptide isolated from rat, pig and human pituitaries has been discussed. The close resemblance of the latter to the C-terminal portion of ACTH, and of α-MSH to the N-terminal part of the ACTH molecule, suggest that α-MSH and the C-terminal peptide are the result of intracellular cleavage of ACTH. Implications of this mechanism have been considered.

INTRODUCTION

SECTION 1

THE VERTEBRATE PITUITARY

All vertebrates have a well-developed pituitary gland consisting of a neural component, the neurohypophysis, and an ectodermal component, the adenohypophysis (Dodd, 1963; Wingstrand, 1966) and its basic structure is remarkably uniform throughout the vertebrate series. The hormones produced by the various parts of the pituitary afford the most stable basis on which to establish homologies (Dodd, 1963). Thus, available evidence points to a constancy in the presence of the major pituitary hormones: the hypothalamo-neurohypophysial system produces octapeptides like oxytocin; the pars intermedia produces the melanocyte stimulating hormones and the pars distalis produces the trophic hormones. The nomenclature employed for these parts of the pituitary in the different vertebrate classes is shown in Table 1. Aspects of the individual components of the adenohypophysis will be considered briefly:

Pars distalis: Lampreys have a pituitary gland which is divided into three distinct serially arranged regions, the anterior and middle of which can be equated with the pars distalis of higher forms on the criterion of embryological origin. In the elasmobranchs, the adenohypophysis gives rise to several projections which, in the adult, are termed the 'ventral, rostral and neurointermediate' lobes. The rostral and ventral lobes together constitute the pars distalis, with the ventral lobe being the probable source of the glycoprotein hormones and the rostral lobe the source of ACTH (de Roos & de Roos, 1967). In the

bony fishes, the pituitary consists of serially arranged regions analogous to the situation in the lamprey. There is no distinct division between the three, but the anterior two, termed pro- and meso- adenohypophyses, undoubtedly represent the pars distalis of higher forms. The amphibian pituitary does not contain a separate pro- and meso- adenohypophysis, but possesses an undivided pars distalis which is separated by a cleft from the pars intermedia. Reptiles, birds and mammals, have a discrete pars distalis of variable form.

Pars intermedia: In lampreys and bony fishes, the pars intermedia comprises the most posterior of the three pituitary regions, termed the meta-adenohypophysis. It is recognised by its relation to the pars nervosa and its content of MSH (Dodd, 1963). The meta-adenohypophysis of the lamprey is a plate-like structure, underlying and separated from the pars nervosa by a blood space. There is no penetration of the meta-adenohypophysis by nerve fibres from the pars nervosa. In the elasmobranchs, there is extensive intermingling of pars nervosa and pars intermedia tissue, which justifies the term 'neurointermediate lobe' for this portion of the gland. In bony fishes, all parts of the adenohypophysis are intermingled with the nervous tissue, but this is greatest in the meta-adenohypophyseal region. The pars intermedia of amphibians and reptiles is generally discrete. In birds, it appears to be absent, although it has been suggested that pars intermedia cells may have been incorporated into the pars distalis. In certain mammalian pituitaries, there is an analogous situation (e.g. man and whales), but most species possess two distinct lobes, which may be

separated from each other by a cleft (e.g. rat, pig). In those mammals in which there is a distinct pituitary cleft, it is simple to dissect out the two lobes; the posterior lobe containing the pars intermedia attached to the pars nervosa, and the anterior lobe containing pars distalis tissue only.

<table>
<tr><th>MAMMALS,
REPTILES,
AMPHIBIANS</th><th>BIRDS</th><th>TELEOSTS</th><th>ELASMOBRANCHS</th><th>CYCLOSTOMES</th></tr>
<tr><td>PARS DISTALIS</td><td>PARS DISTALIS</td><td>PRO- PLUS MESO-
ADENOHYPOPHYSES</td><td>ROSTRAL AND
VENTRAL LOBES
(PARS DISTALIS)</td><td>PRO- PLUS MESO-
ADENOHYPOPHYSES</td></tr>
<tr><td>PARS INTERMEDIA</td><td>(ABSENT?)</td><td rowspan="2">META-
ADENOHYPOHYSIS

(MINGLED WITH
PARS NERVOSA
TISSUE.)</td><td rowspan="2">NEUROINTERMEDIATE
LOBE
(MINGLED PARS
INTERMEDIA AND
PARS NERVOSA.)</td><td>META-
ADENOHYPOPHYSIS</td></tr>
<tr><td>PARS NERVOSA</td><td>PARS NERVOSA</td><td>PARS NERVOSA</td></tr>
</table>

Table 1 : Nomenclature of the different pituitary components in all the vertebrate classes ; homologies are indicated along the horizontal axis.

SECTION 2

ACTH AND RELATED PEPTIDES IN MAMMALS

a) DEFINITION AND STRUCTURE OF ACTH

(i) Definition

Adrenocorticotrophin (ACTH) is defined (Evans *et al.*, 1966) as a factor produced by the adenohypophysis that is necessary for the maintenance of the bulk of the cells of the adrenal cortex and the regulation of their secretory activity in vertebrate animals after birth.

(ii) Isolation and characterisation

The dependence of the zona fasciculata and reticularis of the adrenal on the pituitary gland was first demonstrated by Smith (1930), who found marked adrenal atrophy in hypophysectomised rats. This change could be prevented by intramuscular implants of fresh rat pituitaries and by injections of extracts of bovine anterior hypophysis. This and further work confirmed the presence of a distinct pituitary hormone with a trophic influence on the adrenal cortex.

The corticotrophic activity was found to be more difficult to extract than other pituitary hormones and the classical methods of protein fractionation proved unsatisfactory (Collip *et al.*, 1933; Moon, 1937). Furthermore, no good quantative assay was available to monitor these procedures. ACTH was found to have greater heat and acid stability than the other pituitary hormones and a considerable advance was made by Lyons (1937), who used a concentrated acid-acetone mixture for pituitary extraction. Although designed for prolactin purification, this method has been employed extensively for the initial extraction of ACTH. It was used by Li and his colleagues

(1943) and by Sayers and his colleagues (1943) for the preparation of ACTH from sheep and pig pituitaries which they considered free from contamination by other pituitary hormones and homogeneous on electrophoresis, sedimentation and solubility tests. Its biological potency of approximately 1 iu/mg was rapidly destroyed by tryptic digestion whereas it was markedly stable to peptic digestion. Subsequently, improved methods of separation (counter-current distribution and chromatography) showed these preparations to be heterogeneous (Cortis-Jones et al., 1950).

A major improvement in the purification of ACTH followed the discovery that cellulose and oxycellulose selectively adsorb ACTH from protein extracts (Payne et al., 1950; Astwood et al., 1951). After extraction of porcine pituitary glands with glacial acetic acid at 70° the material had a potency of 2 iu/mg. Adsorption on to oxycellulose followed by elution with 0.1N HCl gave a preparation with a potency of approximately 80 iu/mg. Characterisation of the product showed it was heterogeneous, water soluble, non-volatile and of relatively small molecular weight. Enzyme digestion suggested it was a peptide, and tyrosine, tryptophan and arginine residues were identified. Its behaviour on cation exchange systems suggested the presence of strongly basic residues.

Further purification of ACTH concentrates has been achieved by ion exchange chromatography (Amberlite IRC 50) for pig (White and Fierce, 1953; Dixon & Stack-Dunne, 1955), sheep (Li et al., 1954) and ox (Li & Dixon, 1956) hormones. Counter-current distribution has been used for the final purification of sheep (Li et al., 1955), ox

(Li & Dixon, 1956) and pig (Shepherd *et al*., 1956a) ACTH. The last study yielded several fractions with high ACTH activity. The most abundant (termed β-corticotrophin) was re-purified by counter-current distribution and used for structural studies (Bell, 1954; Howard *et al*., 1955; Shepherd *et al*., 1956b). More recently, substituted celluloses have become available and have been applied to the purification of ACTH from pig (Farmer, 1959), man (Lee *et al*., 1961a), and sheep (Pickering *et al*., 1963; Birk & Li, 1964a). Large scale purification of human and porcine ACTH has recently been achieved by a combination of gel filtration on Sephadex and CM-cellulose chromatography (Lerner *et al*., 1968).

(iii) Structure

Following the purification of the major ACTH component in sheep, pig and ox pituitaries, extensive structural studies established their primary amino acid sequence (Bell, 1954; Howard *et al*., 1955; Shepherd *et al*., 1956b for porcine ACTH: Li *et al*., 1961 for bovine ACTH and Lee *et al*., 1961a, for C-terminal human ACTH). Revised amino acid sequences have recently been proposed for porcine and human ACTH (Riniker *et al*., 1972), and ovine and bovine ACTH (Li, 1972). Total synthesis of the porcine hormone was achieved in 1963 (Schwyzer & Sieber). ACTH comprises a single polypeptide chain containing 39 amino acids with free NH_2 and COOH-terminal residues. It is thus the smallest hormone so far recognised in the anterior lobe of the pituitary. Ovine, bovine and human corticotrophins have the same amino acid sequence, though the amide content of some preparations is still uncertain. Porcine ACTH contains one less serine

and an additional leucine compared to the other characterised corticotrophins (Table 2). ACTH does not seem to have significant secondary or tertiary structure, since conformational changes occur readily with changes in temperature and ionic strength (Botré & Solinas, 1964), as confirmed by dialysis studies (Craig et al., 1965, 1972).

(iv) Nomenclature

The nomenclature proposed by Li (1959a) for ACTH and ACTH-like peptides is used in this thesis. Intact adrenocorticotrophin is termed α-ACTH with the particular species of origin being designated by a subscript. Thus α_s, α_p, α_b and α_h-ACTH refer to the hormones derived from the unhydrolysed extract of sheep, pig, beef and human pituitaries respectively. If more than one active component is derived from such an extract they are labelled sequentially e.g. α_h, β_h, γ_h, etc., for human series. Peptide fragments related to ACTH are designated with a superscript. Thus a peptide with the same amino acid sequence as that in positions 17 to 39 of the pig molecule is called α_p^{17-39} ACTH. Where the sequence is common to all species, so far investigated, the subscript can be omitted, e.g. α^{1-24}. The entire synthetic molecule is referred to as synthetic α_p ACTH to distinguish it from the natural hormone.

b) DEFINITION AND STRUCTURE OF MSH

(i) Definition

The pituitary gland stores peptides that disperse melanin pigment within the chromatophores found in the dermis and epidermis of most species of cold-blooded vertebrates. The name melanocyte-stimulating hormone (MSH) is used by most authors, but the hormone has also been

called melanophore-expanding or melanophore-dispersing hormone (MEH, MDH) or intermedin.

(ii) Isolation and characterisation

During the period 1916-1924, it was established by surgical extirpation and injection of pituitary extracts, that melanocyte activity in frogs is controlled directly by a hormone from the intermediate lobe (Waring, 1963). In the 1930's similar observations were made using other groups of vertebrates, including mammals. Numerous attempts were made, during the late thirties and forties, to extract the active principle in pure form (Zondek & Krohn, 1932; Stehle, 1936; Landgrebe & Waring, 1941). The first major success was achieved by oxycellulose adsorption of MSH from relatively crude extracts of pig pituitaries (Landgrebe & Mitchell, 1954; Lerner & Lee, 1955). Lerner and Lee (1955) further purified their MSH preparation by counter current distribution and paper electrophoresis and obtained a virtually pure polypeptide with a basic charge. In contrast, Porath and his colleagues (1955) and, subsequently, Benfey and Purvis (1955) and Geschwind and his colleagues (1956) obtained acidic melanocyte stimulating peptides from pig pituitaries. The apparent discrepancy was resolved by Lee and Lerner (1956) who showed that pig pituitaries contained both peptides. Their separation was achieved, inadvertently, by an acetone precipitation step that had been introduced after the elution of material from the oxycellulose. The highly basic polypeptide which Lerner and Lee originally isolated was given the name α-MSH, while the acidic polypeptide isolated by other workers was called β-MSH.

α-MSH was subsequently found in bovine (Lo *et al.*, 1961), ovine (Lee *et al.*, 1963), equine (Dixon & Li, 1960) and macaque pituitaries (Lee *et al.*, 1961b) and β-MSH in bovine (Geschwind *et al.*, 1957a, b), ovine (Lee *et al.*, 1963), human (Dixon, 1960), equine (Dixon & Li, 1961) and macaque (Lee *et al.*, 1961b) pituitaries.

(iii) Structure

The structure of porcine β-MSH was first published by Harris and Roos (1956) and independently by Geschwind and his colleagues (1956). Subsequently, the primary structures of the following peptides were determined: bovine α-MSH (Li, 1959b) and β-MSH (Geschwind *et al.*, 1957a, b), ovine α-MSH and β-MSH (Lee *et al.*, 1963), equine α-MSH (Dixon & Li, 1960) and β-MSH (Dixon & Li, 1961), monkey α-MSH and β-MSH (Lee *et al.*, 1961b), human β-MSH (Harris, 1959a) and porcine α-MSH (Harris & Lerner, 1957; Harris, 1959b)

α-MSH:- The structure of α-MSH has been the same in all mammalian species studied (Table 2). It is a tridecapeptide containing an amino acid sequence identical to the 1 to 13 sequence of ACTH but with an acetyl group blocking the terminal amino group, and an amide group blocking the terminal carboxyl group.

β-MSH:- β-MSH comprises 18 amino acids in the pig, sheep, ox, monkey and horse. In man there are four extra amino acids at the N-terminus (Table 2). Differences between the identified peptides are small and all possess a sequence of seven amino acids identical to that of α^{4-10}ACTH which relates them to corticotrophin and to α-MSH. Substitutions are found at positions 2, 6 and 16.

Peptide	Sequence	Species
ACTH	(1) H-SER-TYR-SER-MET-GLU-HIS-PHE-ARG-TRP-GLY-LYS-PRO-VAL(13)-GLY-LYS-LYS-ARG-ARG(18)-PRO-VAL-LYS-VAL-TYR-PRO(24)-ASN(25)-GLY-ALA-GLU-ASP-GLU-SER-ALA-GLU-ALA-PHE-PRO-LEU-GLU-PHE(39)-OH	human
	ASP ... SER ... GLN	sheep
	ASN ... SER ... GLN	ox
	ASN ... LEU ... GLU	pig
αMSH	(1) CH_3CO-SER-TYR-SER-MET-GLU-HIS-PHE-ARG-TRP-GLY-LYS-PRO-VAL(13)-NH_2	Sheep, ox, pig, horse, monkey
βMSH	(1) H-ASP-SER-GLY-PRO-TYR-LYS-MET-GLU-HIS-PHE-ARG-TRP-GLY-SER-PRO-PRO-LYS-ASP(18)-OH	Sheep, ox
	GLU ... LYS ... PRO	pig
	GLU ... LYS ... ARG	horse
	GLU ... ARG ... PRO	monkey
	(1) H-ALA-GLU-LYS-LYS ... GLU ... ARG ... PRO ... (22)	human
βLPH	(1) (NH_2)....ALA(37)-GLU-LYS-LYS-ASP-SER-GLY-PRO-TYR-LYS-MET-GLU-HIS-PHE-ARG-TRP-GLY-SER-PRO-PRO-LYS-ASP-LYS-ARG(60)....(COOH)(90)	sheep
	GLU ... LYS ... PRO ... (91)	pig
	Not determined .... LYS ... GLU ... ARG ... PRO ... ([illegible]1)	human
γLPH	(1) (NH_2) ....ALA(37)-GLU-LYS-LYS-ASP-SER-GLY-PRO-TYR-LYS-MET-GLU-HIS-PHE-ARG-TRP-GLY-SER-PRO-PRO-LYS-ASP(58)-OH	sheep
	GLU ... LYS ... PRO	pig

Table 2 : Amino acid sequences of mammalian pituitary ACTH-like peptides.

Monkey β-MSH has an identical sequence to the 5 to 22 portion of human β-MSH. It has been claimed that 'bovine' (β-Ser_2-MSH) and 'porcine' (β-Glu_2-MSH) β-MSHs can be found together in individual ovine, bovine and porcine pituitaries (Burgers, 1961; Geschwind, 1966).

c) OTHER ACTH-LIKE PEPTIDES

(i) Lipotrophic hormones

During the investigation of a simplified procedure for isolating ACTH from sheep pituitary glands, Birk and Li (1964b) obtained a peptide with lipolytic properties, which was chemically distinct from ACTH and other adenohypophyseal peptides. They designated this peptide 'lipotrophic hormone' (LPH) and the complete amino acid sequence of one LPH, now termed β-LPH, was published by Li and his colleagues in 1965. In the course of purifying this peptide a further peptide with lipolytic activity was obtained, and its structure determined (Chrétien & Li, 1967). This second peptide was termed γ-LPH. Subsequently, peptides similar in amino acid composition, sequence and biological properties have been isolated from ox (Lohmar & Li, 1967), pig (Gráf & Cseh, 1968; Gráf et al., 1970) and human (Cseh et al., 1968) pituitary glands.

Sheep (Li et al., 1965) and pig (Gráf et al., 1971) β-LPHs comprise ninety and ninety-one amino acid residues respectively (Table 2), and include the sequence of the corresponding β-MSH in positions 41 and 58 (i.e. β-Ser_2-MSH in sheep, and β-Glu_2-MSH in pig). The extra four N-terminal amino acids of human β-MSH are also found in sheep lipotrophin in position 37 to 40. Only part of the human β-LPH molecule has been sequenced

(Cseh et al., 1972), and at least a portion of the sequence of human β-MSH has been shown to occupy positions 40 to 58 of this molecule. The γ-LPHs from sheep (Chrétien & Li, 1967) and pig (Gráf et al., 1970) are identical in sequence with the portions 1 to 58 of their corresponding β-LPHs, and therefore, contain at their C-terminals the complete sequences of β-Ser_2-MSH and β-Glu_2-MSH respectively (Table 2).

(ii) Corticotrophin releasing factors similar to α-MSH

Several peptides with corticotrophin releasing activity were isolated from posterior pituitary lobe extracts in the early sixties. A peptide α_1-CRF contained all the amino acids of α-MSH plus threonine, alanine and leucine. It had the heptapeptide sequence common to all known types of mammalian MSH, ACTH and LPH and exhibited melanotrophic and corticotrophic activity (Guillemin et al., 1960). α_2-CRF had an identical sequence to α-MSH but the α-amino acid group was blocked in some way other than by an acetyl group (Schally et al., 1962).

(iii) 'Precorticotrophin' and 'Big-ACTH'

Dasgupta and Young (1958) obtained an alkaline extract of bovine pituitaries in which ACTH activity could be generated by acidification. It was suggested that activity might result from the release of ACTH from a precursor molecule. Yalow and Berson (1971) also demonstrated a material with ACTH immunoactivity, but of larger molecular size, in human plasma, pituitaries and tumour extracts.

(iv) Other pituitary peptides

Electrophoretic and immunological methods

indicated the presence in porcine, ovine and bovine pituitary tissue of only β-Ser_2-MSH, β-Glu_2-MSH and α-MSH (Burgers, 1961; Geschwind, 1966; Shapiro *et al.*, 1972). However, some forms of MSH in rodent (rat, guinea pig, etc.) pituitaries did not behave electrophoretically or immunologically like the known peptides (Thody, 1969 ; Shapiro *et al.*, 1972).

(v) Tumour peptides

The presence of ACTH in non-pituitary tumours in man was first described by Meador and his colleagues (1962). Immunoreactive α-MSH (Abe *et al.*, 1967a) and β-MSH (Abe *et al.*, 1969) were subsequently demonstrated in tumour extracts. Two fragments of the ACTH molecule have been tentatively identified by immunological and physicochemical procedures in certain tumours (Orth *et al.*, 1968, 1971; Shapiro *et al.*, 1971) and materials resembling ACTH in amino acid composition have been isolated from human non-pituitary tumours (Upton & Amatruda, 1971), and a mouse pituitary tumour (Canfield *et al.*, 1970).

d) BIOLOGICAL AND IMMUNOLOGICAL ACTIVITIES OF ACTH AND MSH

The role of ACTH in maintenance of adrenal growth, adrenal ascorbic acid depletion and steroidogenesis has been established in a large number of species. Extra-adrenal effects of ACTH, such as melanocyte stimulation and lipolysis have been established in only a limited number of mammals (Engel & Lebovitz, 1966). The physiological significance of these actions is doubtful, since, in most situations, normal circulating levels of ACTH are too low to exert any activity outside the adrenal.

A major role of α-MSH and β-MSH in fish, amphibians and reptiles is that of controlling skin colour. In mammals, however, there is no such role, despite mammalian α-MSH being very potent in its action on the melanphores of these lower vertebrates (Lowry & Chadwick, 1970b). Abnormally high concentrations of α-MSH will cause skin darkening in humans, but racial variations in pigmentation in man are not related to secreted MSH, (Schizume & Lerner, 1954). A number of possible roles for MSH in mammals are listed in Appendix 1.

(i) Bioassay of ACTH

Bioassays for ACTH developed and improved over a 40 year period. Among the first, were those based upon changes in adrenal weight (Collip et al., 1933; Moon, 1937; Simpson et al., 1943), but substances other than ACTH could affect this parameter (Evans et al., 1966; Segal & Christy, 1968). The discovery that the adrenal ascorbic acid content of the rat adrenal was depleted by ACTH led to the development of a simpler and more specific assay (Sayers et al., 1948) which greatly facilitated the isolation and characterisation of the hormone in the fifties. Now, however, most assays are based on increased steroidogenesis by the adrenal (Saffran & Schally, 1955; Guillemin et al., 1958; Lipscomb & Nelson, 1962; Vernikos - Danellis et al., 1966). The two most recent involve measurement of steroidogenesis by isolated rat adrenal cells (Sayers et al., 1971) and measurement of redox changes in excised guinea-pig adrenals (Chayen et al., 1971). The latter is the most sensitive ACTH assay ever described, being able to detect femtogram amounts. The most important requirement for the type of study described

in this thesis was a high specificity (rather than high sensitivity) and the procedures described by Lipscomb and Nelson (1962) and by Sayers and his colleagues (1971) have been thoroughly characterised in this respect (Ney et al., 1964; Schwyzer et al., 1971). Both have been used in the present study.

(ii) Structural features of importance in ACTH bioassay

There is an extensive literature on the function/structure relationships of ACTH and the ACTH-like peptides, including studies on natural and synthetic peptides (Evans et al., 1966; Harris, 1966; Ney et al., 1964; Schwyzer et al., 1971; Hechter & Braun, 1971) and on chemically modified peptides, (see Evans et al., 1966; Geschwind, 1966). The most important points to arise from these studies are:

All ACTH-like peptides have some steroidogenic activity, exert some effect on melanophores and cause lipolysis in rabbit fat cells, and the amino acid sequence α^{4-10}ACTH is the 'core' responsible for eliciting these activities (Schwyzer et al., 1971; Pickering & Li, 1962; Tanaka et al., 1962). α^{4-10}ACTH, α^{1-10}ACTH and α^{1-13}ACTH possess only minimal corticosteroidogenic activity while α^{1-19}ACTH is half as potent as the full ACTH molecule, suggesting that the basic amino acid region (α^{14-19}) plays a key role in binding the ACTH molecule to its adrenal receptor (Ney et al., 1964). The α^{1-24}portion of ACTH (Bell et al., 1956), a free NH_2-terminal serine (Geschwind & Li, 1958; Waller & Dixon, 1960) and an unoxidised methionine residue (Dedman et al., 1961) are essential for full steroidogenic activity.

An acetylated N-terminal serine increases the MSH activity of ACTH (Harris, 1966) and the optimal length of peptide for melanocyte stimulating activity corresponds to the sequence 1 to 13 of ACTH (i.e. α-MSH).

(iii) Radioimmunoassay of ACTH

Berson and his colleagues (1956) introduced the concept of radioimmunoassay, which is based on the use of a limited concentration of a specific antibody, and on determining the distribution of antigen between the bound and free fractions by means of radioactively labelled antigen.

Radioimmunoassays for ACTH were first described by Felber (1963) and subsequently by other workers (Demura et al., 1966; Orth et al., 1968; Landon & Greenwood, 1968; Berson & Yalow, 1968; Donald, 1968; Matsukura et al., 1971; Rees et al., 1971).

It is proposed to discuss briefly certain aspects of standardisation and antiserum specificity relevant to ACTH radioimmunoassay:-

Quantitation in any assay depends on comparing the response given by the test material with that by a reference preparation. Reference porcine corticotrophin is readily available, since it is effective therapeutically, and the major international standard for ACTH bioassay is partially purified porcine ACTH (IIIrd I.S., Bangham et al., 1962). This has been employed in radioimmunoassay (Rees et al., 1971), but the majority of workers, concerned more with the clinical application of the assay, have preferred a human ACTH standard. Supplies of human material are limited, however, and therefore different workers have been forced to employ

a variety of preparations. These have included both natural (e.g. Lerner, Upton and Li preparations) and synthetic (CIBA, Ferring, Armour) human ACTHs.

The availability of high avidity antibodies is the single most important factor in setting up a sensitive immunoassay (Hurn & Landon, 1971). ACTH is poorly immunogenic, and extensive immunisation programmes have been required to raise satisfactory antisera (Berson & Yalow, 1968; Orth et al., 1968). The majority have been raised against natural or synthetic human and porcine ACTHs or synthetic α^{1-24}ACTH, suspended in Freunds adjuvant, adsorbed onto carbon microparticles or coupled covalently to a larger molecule.

Binding involves only part(s) of the ACTH molecule termed the antigenic determinant(s), which can be located by inhibition studies with synthetic or natural ACTH fragments (Orth et al., 1968; Berson & Yalow, 1968; Landon, 1968; Felber & Aubert, 1968; Aubert & Felber, 1969). Antisera have been raised which are predominantly directed at the α^{25-39}(C-terminal) portion of the ACTH molecule, which therefore cross-react with biologically inactivated α^{1-39}ACTH (Imura et al., 1965) and C-terminal fragments of ACTH (Besser et al., 1971). Antisera have also been raised which are directed specifically at sequences within the α^{1-24}(N-terminal) portion (Orth et al., 1968; Landon & Greenwood, 1968; Rees et al., 1971), or at sequences within both portions of the ACTH molecule (Berson & Yalow, 1968; Ratcliffe et al., 1972).

(iv) Structural features of importance in ACTH radioimmunoassay

The C-terminal portion of the ACTH molecule is relatively more immunogenic than the N-terminal, probably

because of its species differences (Landon, 1968). Since this portion differs from that required for biological activity, a dissociation between immunological and biological activity is possible. Such a dissociation is also possible, even with antisera directed towards the biologically active portion of the molecule since, for example, oxidation of the methionine residue causes loss of biological activity, without loss of immunoactivity. Iodination may alter both the immunological and biological activity of ACTH (Leftkowitz et al., 1970).

e) LOCALISATION OF ACTH IN THE MAMMALIAN PITUITARY

(i) Pars distalis

Most of the bioactive and immunoactive ACTH in the rat pituitary is present in the pars distalis, and present evidence suggests that 'corticotrophs' are chromophobic stellate cells scattered throughout and comprising a small percentage of the cells of the pars distalis. They stain specifically with anti-ACTH sera (Table 3), show ultrastructural changes following adrenalectomy, and incorporate tritiated glycine (Siperstein, 1963) which implicate them in ACTH production and release (Siperstein & Miller, 1970).

(ii) ACTH in the pars intermedia of the pituitary

It has been suggested that the pars intermedia of the rat pituitary may also be involved in ACTH secretion since (a) the posterior lobes appear to contain appreciable amounts of bioactive ACTH (Miahle-Voloss, 1958; Rochefort et al., 1959; Smelik et al., 1962) (b) the morphology of the cells in the pars intermedia is altered by factors affecting ACTH secretion (e.g. adrenalectomy) (Kobayashi, 1968) (c) certain cells of the pars intermedia resemble the 'corticotrophs' of the pars distalis (Klein

et al., 1970; Porte et al., 1971)(d)adrenalectomy accelerates the incorporation of tritiated thymidine into pars intermedia cells (Gosbee et al., 1970)(e)the pars intermedia cells can be immunostained with anti-ACTH sera (Table 3). Nonetheless, controversy remains over the presence or absence of ACTH in pars intermedia cells. Firstly, it is not clear whether the major proportion of bioactive ACTH found in posterior lobe extracts is derived from the pars intermedia or pars nervosa. Secondly, ultrastructural and autoradiographical studies suggest, but do not prove, the involvement of the pars intermedia cells in ACTH secretion. Thirdly, evidence derived from immunohistological studies is difficult to evaluate in the absence of sufficient information on the specificities of the antisera employed and ignorance concerning the nature of the ACTH-like peptides in the rat pituitary. For example, in three reports describing the immunohistological localisation of α^{1-24}ACTH antisera in the rat pars intermedia, it was thought likely that cross-reaction with α-MSH had occurred (Hess et al., 1968; Nakane, 1970; Herlant et al., 1972), although Breustedt (1968), in a similar report, thought it unlikely. Phifer and Spicer (1970) took positive steps to exclude the possibility of cross-reaction with α-MSH, by raising their antiserum to a synthetic peptide identical in sequence with the C-terminal portion of the ACTH molecule, and McGarry and Beck (1970) demonstrated that the α_p^{1-39}antisera they employed did not cross-react in immunological tests with α-MSH. In the rat pituitary, however, Baker and his colleagues (1970), also using a α_p^{1-39} antiserum, failed to obtain similar results. Furthermore, although Phifer and Spicer

(1970) reported a strong reaction of their α_p^{17-39} antiserum with pars intermedia cells in the pig pituitary, Dubois (1972), using a well-characterised, specific ACTH antiserum, was unable to demonstrate any reaction at all.

The immunohistological evidence for the localisation of ACTH in the mammalian pars intermedia is thus uncertain and contradictory. If, however, the pars intermedia of pig and rat pituitaries contained a peptide with a sequence in common with the N-terminal part of ACTH (i.e. α-MSH) and a second with a sequence in common with the C-terminal part, then the apparently disparate results could be reconciled. Evidence for this postulate is presented in this thesis.

f) LOCALISATION OF MSH IN THE MAMMALIAN PITUITARY

There is considerable evidence that the melanocyte-stimulating peptides are produced in the intermediate lobe of the pituitary. The bulk of bioactive MSH in the rat pituitary is in the posterior lobe (Thody, 1969; Shapiro et al., 1972). α-MSH has been demonstrated by immunohistological means in pars intermedia cells (Table 3) of both the rat and pig and, in the latter, both α-MSH and β-MSH can be detected by immunohistological means in the same cells (Dubois, 1972). The uniform staining of the pars intermedia suggests that all the cells are of one functional type.

β-MSH antisera have also been shown to immunostain ACTH-containing cells in the pars distalis of the pig (Dubois, 1972) and human (Phifer et al., 1970) pituitary. Cross-reaction of the β-MSH antisera with ACTH is unlikely and the possibility of cross reaction with the

lipotrophic hormones was not mentioned in the interpretation of these results. Such an explanation seems feasible, in view of the common sequence of amino acids shared by β-MSH and LPHs (Table 2).

ANTISERUM RAISED TO:	CROSS-REACTION WITH: αMSH	βMSH	α^{1-24} ACTH	α^{1-39} ACTH.	IMMUNOSTAINS CELLS IN: P.distalis	P.intermedia
					A. RAT	
(1) α^{1-24} ACTH	?	?	+	+	+	+
(2) α^{1-24} ACTH	?	?	+	?	+	+
(3) α^{1-24} ACTH	?	?	+	?	+	+
(4) α^{1-24} ACTH	?	?	+	?	+	+
(5) α_p^{1-39} ACTH	–	?	?	+	+	+
(6) α_p^{1-39} ACTH	?	?	?	+	+	–
(7) α_p^{17-39} ACTH	–	–	–	+	+	+
(8) αMSH	+	?	?	?	–	+
					B. PIG	
(9) α^{1-24} ACTH	+	?	+	?	+	+
(10) α^{1-24} ACTH	–	–	+	+	+	–
(11) α_p^{17-39} ACTH	–	–	–	+	+	+
(12) αMSH	+	–	–	–	–	+
(13) βMSH	–	+	–	–	(+)	+

References

(1)(8),Hess et al.(1968) ; (2), Breustedt (1968) ; (3),Nakane(1970) (4)(9),Herlant et al.(1972) ; (5),McGarry & Beck (1970) ; (6),Baker et al.(1970) ; (7)(11), Phifer & Spicer(1970) ; (10)(12)(13), Dubois(1972) .

+ , positive immunostaining , or proven cross-reaction with peptide.

– , negative immunostaining , or proven non-cross-reaction with peptide.

(+) , attenuated immunostaining.

? , cross-reaction with peptide not tested.

Table 3 : Published reports on immunohistological localisation of ACTH-like peptides in rat and pig pituitaries.

SECTION 3

ACTH AND RELATED PEPTIDES IN NON-MAMMALIAN VERTEBRATES

a) THE VERTEBRATE PITUITARY-ADRENAL AXIS AND ACTH

There is a large and comprehensive literature relating to the adrenals and adrenocorticoid secretions of non-mammalian vertebrates (Chester Jones, 1957; Nandi, 1967). All vertebrates possess adrenal tissue. Bird, reptile and amphibian adrenals secrete predominantly corticosterone. Teleost adrenals mainly secrete cortisol and elasmobranch adrenals secrete an unusual steroid, 1- α - hydroxycorticosterone. Cyclostome adrenals, which comprise scattered sudanophilic cells in the walls of the cardinal veins, do not produce corticosteroids *in vitro* (Weisbart & Idler, 1970) and workers have failed to detect circulating plasma corticosteroids using refined steroid measurement techniques (Weisbart & Idler, 1970; Larsen, 1969).

(i) Identification of adrenocorticotrophic hormone in vertebrates

There have been numerous observations, in a wide range of non-mammalian vertebrates, that hypophysectomy causes adrenal atrophy and cessation of corticosteroid secretion, indicating the existence of an adrenal-controlling pituitary factor (Appendix 2). In the lamprey (Hardisty, 1972) and the dogfish *Scyliorhinus caniculatus* (Dodd, In Hoar, 1966), however, hypophysectomy does not cause adrenal atrophy. This suggests an absence of pituitary control, although it may also reflect a seasonal or temperature dependence of the adrenal upon ACTH as observed in amphibians (Chester Jones, 1957) and some

fishes and reptiles (e.g. Licht & Bradshaw, 1969), or alternatively an extra-hypophysial source of ACTH (as shown for birds - Miahle & Koch, 1969).

(ii) Nature of non-mammalian vertebrate corticotrophins

Present knowledge concerning the corticotrophic hormones in non-mammalian vertebrates relies on a comparative approach. This entails studying the effect of a pituitary extract or purified ACTH from one animal on the adrenal of another. The specificity of the receptor sites on mammalian adrenal cells, with reference to a particular amino acid sequence, implies that for a vertebrate pituitary extract to stimulate a mammalian adrenal it must contain a substance with an identical or very similar sequence to the mammalian hormone. Similarly, if mammalian ACTH stimulates the adrenals of lower vertebrates, this also implies that the non-mammalian vertebrate possesses a corticotrophin with a similar structure to that of mammalian ACTH. The extensive literature (Appendix 2) gives an overall impression that the biologically active portion of the ACTH molecule is similar throughout the vertebrates. It should be noted, however, that many of the reports have employed inadequate experimental controls, and in many instances pituitary extracts have been injected into non-hypophysectomised animals, where the release of endogenous ACTH cannot be excluded. Finally, many studies have been based upon uncharacterised or non-specific assay methods.

A better approach to identifying the structure of vertebrate corticotrophins is by using immunological techniques (e.g. immunoprecipitation or radioimmunoassay) with their high degree of specificity and sensitivity.

Shapiro and his colleagues (1972) have employed radioimmunoassays for the MSHs and ACTH to characterise these peptides in a range of vertebrates. The ACTH antibody used in their study cross-reacted with the N-terminal, biologically active portion of the molecule, and pituitary extracts from a flounder, bullfrog and chicken were found to contain cross-reactive material.

Apart from this instance, no other radioimmunological studies on ACTH have been reported. This is unfortunate, since the technique is ideally suited to investigate the structural similarities of the non-biologically active portions of vertebrate corticotrophins.

b) EVIDENCE FOR RELATED PITUITARY HORMONES

The presence of MSH activity in non-mammalian vertebrate pituitaries has been well documented. Lanzing (1954) showed that lamprey pituitaries contained a substance that would blacken frogs. Burgers (1963) provided electrophoretic evidence for three MSHs in *Rana catesbiana* and *Anolis carolensis*, and two in *Gadus morhua* (see also Geschwind, 1966). In terms of electrophoretic mobility, the MSHs corresponded to either mammalian α-MSH or β-MSHs with the exception of one MSH in the bullfrog and three in the chameleon (*Anolis c.*). Preslock and Brinkley (1970) also identified three MSHs in *Rana pipiens* pituitary extracts and Dickhoff (1972) identified five in *Rana catesbiana*.

Shapiro and his colleagues (1972) investigated vertebrate pituitaries using a radioimmunoassay for α-MSH and β-MSH. In only two of the ten species they studied (pig and ox) could they account for the biological MSH activity in terms of the known hormones. All the

extracts contained a material which cross-reacted with the α-MSH antiserum, but none of the non-mammalian extracts, or those of rodents, contained material that cross-reacted with the β-MSH antiserum. It was concluded that, in these species, the structure of the major melanocyte-stimulating hormone is different from any other that had been characterised previously.

Recently, Lowry and Chadwick (1970a) have isolated two MSH molecules of much lower potency than mammalian α-MSH from the spiny dogfish *Squalus acanthias*. They contain eleven and twelve amino acid residues respectively, which are identical to those of mammalian α-MSH except that methionine is substituted for the C-terminal valine, the N-terminal amino acid residue is not acetylated and half of the C-terminal methionine residues are amidated. Love and Pickering (1972) have reported on the isolation of a β-MSH like peptide from the spotted dogfish *Scyliorhinus caniculatus*.

c) LOCALISATION OF CORTICOTROPHIC AND MELANOTROPHIC ACTIVITY IN VERTEBRATE PITUITARIES.

Knowledge of the pituitary origin of the corticotrophic and melanotrophic hormones is of considerable help in distinguishing between the effects of the two hormones in assays since, in high concentrations, ACTH has appreciable melanocyte stimulating activity and MSH has appreciable corticotrophic activity. The evidence for their localisation, derived from assay and histology, is tabulated in Appendix 3. It is apparent that throughout the elasmobranch, teleost, amphibian, reptile and mammalian classes, there is constant separation of the

bulk of ACTH activity into the pars distalis (or homologue) and of MSH activity into the pars intermedia (or homologue). In the cyclostomes, the situation with regard to MSH activity is identical, but the localisation of ACTH activity is uncertain. In birds, the bulk of bioactive MSH is present in the same part of the pituitary as the ACTH cells (Kleinholz & Rahn, 1939; Miahle-Voloss & Benoit, 1954), but birds do not possess a distinct pars intermedia and these results suggest either that the pars intermedia cells are mixed with pars distalis cells, the highest concentrations being localised in the rostral tip of the pituitary, or pars intermedia cells are absent, and the MSH activity is due to high concentration of ACTH and/ or LPH.

SECTION 4

PROBLEMS CONCERNING THE EVOLUTION OF THE ACTH-LIKE PEPTIDES

The problems of ACTH evolution have been well reviewed by Geschwind (1967, 1969) and by Lowry and Chadwick, (1970b), who have emphasised the limitations imposed by the lack of knowledge of the structure of ACTHs or MSHs from vertebrates other than a single dog-fish species and several mammals. It is apparent, however, that all of the presently identified peptides fall into two groups, the members of each having points of similarity. Thus ACTH and α-MSH form one group, and β-MSH, β-LPH and γ-LPH form another group. All of these peptides have an intrinsic melanocyte-stimulating action which is derived from the heptapeptide sequence Met-Glu-His-Phe-Arg-Try-Gly- which they hold in common (Table 2).

It has been suggested that the two groups arose originally through gene duplication at an early stage in vertebrate evolution, and the heptapeptide core has been retained, while divergence has occurred in the structure of the rest of the molecule. The identity of sequence between α-MSH and the 1 to 13 portion of ACTH, and β-MSH with the middle portion of the corresponding LPHs in sheep, pig and human pituitaries suggests, however, that ACTH and α-MSH are the products of the same gene, and β-MSH and LPH are the products of another gene. The major objection put forward to this suggestion both by Geshwind (1967) and by Lowry and Chadwick (1970b), was the different sites of origin of ACTH and α-MSH, and LPH and β-MSH. It is the intention in this thesis, however, to present evidence in

support of the concept that α-MSH and ACTH are derived from a single gene. while the close relationship between the two groups of peptides mentioned above, suggests that β-MSH and LPH are similarly derived from a single gene.

METHODS

SECTION 1.

PEPTIDE EXTRACTION AND ISOLATION PROCEDURES

a) TREATMENT OF PITUITARIES

All possible precautions were taken to ensure that pituitary glands were removed from animals immediately after death and frozen at -70°, however, human and dogfish pituitaries obtained from Grimsby were often over 24 hours old. Tissues were weighed after freezing and immediately before extraction.

b) EXTRACTION OF ACTH AND RELATED PEPTIDES FROM TISSUE

The first part of the study required a method suitable for processing a large number of pituitaries from different species for assay - this demanded simplicity, rapidity and reproducibility. The second part of the study required methods suitable for the isolation of ACTH-like peptides from large amounts of tissue and this demanded substantial purification of the peptides in high yield. A number of other factors also had to be considered in devising satisfactory extraction methods:

1) ACTH and MSHs are resistant to strong acids and boiling in acid. Thus it is possible to use vigorous extraction techniques that are inappropriate for some other pituitary hormones.

2) Pituitary tissue is rich in proteolytic enzyme activity (Pickup _et al._, 1971) which must be minimised throughout the extraction procedure. Proteolytic enzyme activity in ox pituitaries is most active at pH 3.8, but can be inactivated by lowering the pH or boiling.

3) Pituitary weight varies greatly in different species (in the present study the weights ranged from 100 μg to 800 mg). Some procedures, especially those involving precipitation, would be unsuitable for small amounts of tissue.

4) Certain solvents, especially those containing concentrated acids, may interfere with assays and should be avoided.

(i) Rapid extraction procedure for pituitary glands

Pituitaries, weighing from 100 μg to 30 mg, were placed in 2 ml capacity polystyrene tubes (LP3, Luckhams, England) together with a few grains of acid washed sand and 0.1N hydrochloric acid. After homogenisation with a glass rod for one minute using a vortex mixer, 0.4ml distilled water was added to make the extracts up to 0.5 ml. After centrifugation for 10 minutes at 3000rpm, the supernatants were removed and portions taken for assay. The remainder of the extracts were frozen at -70°. The final concentration of hydrochloric acid did not interfere with either bioassay or radioimmunoassay.

(ii) Extraction procedures used for purification of ACTH and related peptides

The isolation and purification of ACTH-like peptides demands more selective procedures and several were tried, based on previously published methods. All steps were carried out at 4° unless stated:-

(1) HC1/acetone (Lyons, 1937)

Acetone dried powder was mixed overnight with acid/acetone (2.5 vol HC1/97.5 vol.80% acetone - 20 ml/g powder). After centrifugation and removal of the supernatant, three volumes of cold acetone were added

slowly with stirring. The precipitate was allowed to settle overnight and then collected by centrifugation (3000 rpm, 10 minutes). Fresh human bronchial carcinoid tumour tissue was extracted in a similar fashion, except that concentrated, instead of aqueous acetone was employed in making up the HCl/acetone mixture - three ml. of the mixture was used per g of tissue, the tissue was re-extracted with the same volume of HCl/acetone, and the final precipitate was collected on a sintered glass funnel (Ratcliffe et al., 1972).

(2) TFA/acetone

Acetone dried powder was mixed overnight with TFA/acetone (2 vol. trifluoroacetic acid/98 vol. 80% acetone - 20 ml/g powder). After centrifugation, the supernatant was removed, three volumes of ether added with stirring and left to form a precipitate overnight. The precipitate was collected by centrifugation.

(3) Glacial acetic acid, 5% acetic acid, 0.1N hydrochloric acid or 15% trifluoroacetic acid:-

Acetone dried powder was mixed overnight with either glacial or 5% HAc, 0.1N HCl or 15% TFA (20 ml/g powder). After centrifugation, the supernatants were removed and freeze-dried. Glacial acetic acid extracts were mixed at room temperature. Boiling of 5% HAc and 0.1N HCl extracts was carried out after addition of the acids to the powders. Acetone-dried rat pituitary glands, used in the CLIP and MSH isolation procedures, were homogenised twice in glacial acetic acid and the combined supernatants freeze-dried.

c) PURIFICATION OF ACTH-LIKE PEPTIDES

(i) Reagents

All reagents and solvents were 'analar' grade. Trimethylamine was distilled at 4° from anhydrous trimethylamine containing 10% acetic anhydride (w/v), and stored at 4° as a concentrated aqueous solution (approximately 3M). The trimethylamine was titrated with glacial acetic acid to a pH of 5, and distilled deionised water added to adjust the molarity of the trimethylamine to 1. This solution was diluted approximately with water to the required molarities for column purification.

(ii) Purification and separation of peptides

1) Gel chromatography. Biogel P6 (200-400 mesh) and Biogel P2 (50-100 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) were employed. Columns were packed by allowing a slurry of the previously swollen gel to settle in siliconised glass tubes of constant bore (Jencons) already filled with degassed 5% acetic acid (Lowry & Chadwick, 1970a).

2) Ion-exchange chromatography. DE52 and CM32 cellulose (Whatmans Biochemicals, Maidstone, Kent) were fractionated by a back washing procedure devised by McMartin and Vinter (1969). Microgranular fractions were packed as thick slurries in 500 mM trimethykamine acetate buffer (Me_3NAc), pH 5, in constant bore siliconised glass columns. These were equilibrated in 5 mM Me_3NAc, pH 5, before use and washed with 600 mM Me_3NAc after use.

All flow rates were controlled by Technicon proportioning pumps, and column chromatography was performed at room temperature. Fractions were collected and stored in polystyrene tubes ar 4°. Peptides were detected in column

effluents with a refrigerated (4°) Uvicord II ultra-violet absorbtiometer (LKB Instruments Ltd., South Croydon, London) recording at 280 nm. The detector cell was designed with a 2.5 cm path length (McMartin, Simpson & Thorpe, 1969). Fractions were dried under vacuum from liquid, to minimise freeze drying losses. Gradients were formed by the device shown in Fig.1. Dimensions, buffers and flow rates of all columns used are given in Table 4. In the results section, reference is made to these columns by the number in the left hand column. For example, the DEAE-cellulose column system is referred to as C.S.4.

d) CHEMICAL ANALYSIS OF PEPTIDES

(i) Amino acid analyses

Peptide samples were hydrolysed in 0.2 ml 6N hydrochloric acid in evacuated glass tubes for 16 h at 110° (with a crystal of phenol to prevent tyrosine loss). A Technicon Analyzer was modified to increase sensitivity for 2 - 5 nmol analyses.

(ii) Sequence analysis

N-terminal analysis was carried out by the Edman degradation method of Gray (1967), except that phenyl isothiocyanate was added to the reaction mixture separately instead of it being stored in pyridine. This was used in conjunction with the subtractive procedure, employing amino acid analysis of the remainder of the peptide after removal of the NH_2- terminal amino acid. Pyridine was re-distilled under reduced pressure and stored under nitrogen at -20°. Phenylisothiocyanate (PITC) was redistilled at 1 mm Hg, and the fraction boiling at 55° collected. Trifluoroacetic acid (TFA) was heated under

reflux for 18 hours over CrO_3, and the fraction boiling between 71° and 73° on distillation was collected and stored at room temperature. Butyl acetate was shaken overnight with aqueous 5% $KMnO_4$ (w/v) and washed with water. It was then dried with anhydrous Na_2SO_4 and redistilled. The fraction which boiled at 78° was collected and stored at room temperature.

For C-terminal analysis, carboxypeptidase A was used, with a peptide/enzyme ratio of 50/1, in 0.2M N-ethyl morpholine acetate pH 8.5 at 20°. Carboxypeptidase C digestion was performed in 0.5M sodium citrate buffer (pH 5.3) with a peptide/enzyme ratio of 100/1. Digestions were performed on 1 - 5 nmol aliquots of the peptides and the amino acids released were identified on the Analyzer.

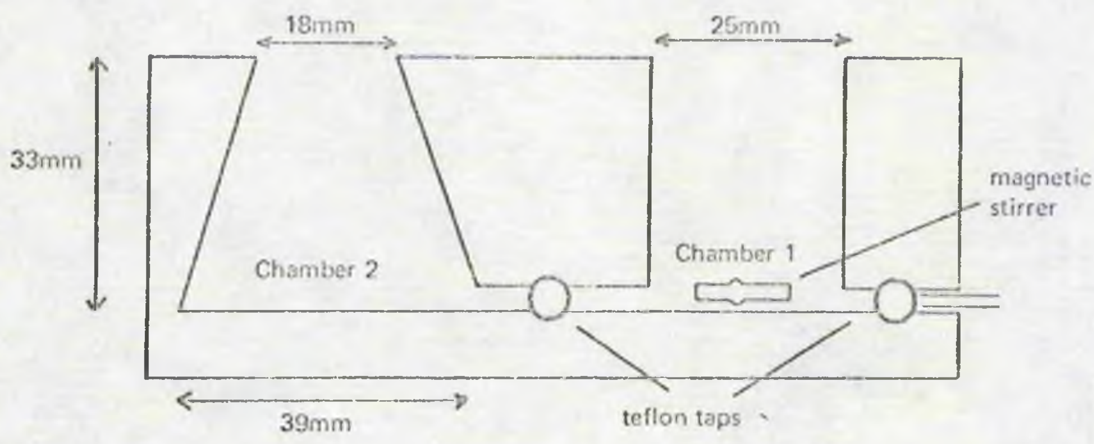


Figure 1: Perspex gradient device shown in cross-section. Cylindrical chamber 1 was filled with 10 mls of starting buffer. Conical chamber 2 was filled with 20 mls of the final buffer.

Column System (C.S.)	Type	Dimensions (cm.) length.	diam.	Buffer	Flow rate (ml/hr)	Sample application volume (ml)
1	Biogel P2 plus Biogel P6 in series .	33 66	2.5 2.5	5°/o HAc	30	10
2	Biogel P6	100	1.0	5°/o HAc	3	0.5
3	Biogel P6	80	1.5	5°/o HAc	6-9	2.0
4	DEAE-cellulose	15	0.4	Me_3NAc(pH5)	1.5	0.5
5	CM - cellulose	10	0.4	Me_3NAc(pH5)	1.5	0.5

Table 4 : Particulars of the chromatography columns employed for the isolation of ACTH-like peptides.

ASSAY PROCEDURES

SECTION 2

a) BIOASSAY

(i) In vivo Lipscomb-Nelson ACTH bioassay

The method of Lipscomb and Nelson (1962), as modified by Ney and his colleagues (1963), was used:- 200 - 250g male Sprague-Dawley rats were anaesthetised with halothane and hypophysectomised by the transaural route. Two hours post-hypophysectomy, the rats were reanaesthetised with an intra-peritoneal dose of Nembutal and the samples (dissolved in saline acidified to pH 3 with hydrochloric acid, containing 0.1% human serum albumin) were injected into the femoral vein. At least two animals were injected with each dose of the unknown tissue extract, and three animals at each dose of standard ACTH. Approximately five minutes after the injection of each sample, the left adrenal gland was exposed, and seven minutes after injection of the sample, a fine needle inserted into the left adrenal vein and blood collected into a 1 ml greased tuberculin syringe. The adrenal vein effluent was collected for three minutes and then transferred to polystyrene tubes. Each blood collection was made up to 3 ml with the acidified saline, and centrifuged. Plasma fluorogenic corticosteroids were measured by a method based on that of Mattingly (1962), but using corticosterone as a standard. The response to two effective dose levels of each unknown tissue extract, and to three doses each of 0.2 and 0.05 mU of the 3rd International Standard (Bangham _et al._, 1962) were compared by a method described by Pugsley (1946) to obtain the potency of the tissue extracts. The minimum detectable amount of ACTH was

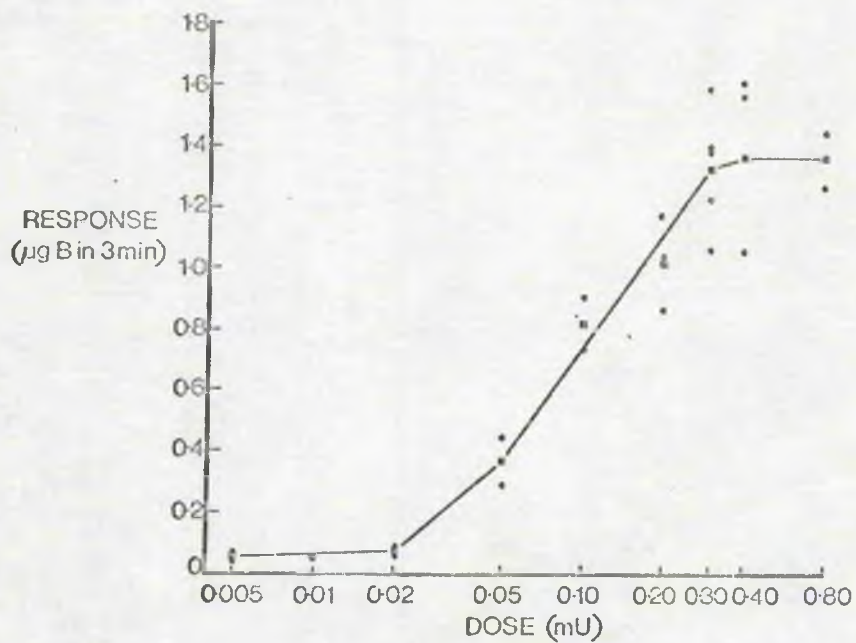


Figure 2: Dose-response curve for 3rd International Standard of ACTH in the Lipscomb-Nelson bioassay.

0.03 mU (Fig. 2). All bioassay values quoted have been converted to gravimetric (weight) terms to simplify comparison with radioimmunoassay data, which employed Lerner Upton natural human ACTH as standard.

(ii) Other bioassays

In several of the chromatographic experiments, fractions were assayed for ACTH bioactivity by the *in vitro* adrenal cell procedure (Sayers *et al.*, 1971) as modified by Lowry, McMartin and Peters (1973). MSH bioactivity was assayed by an in *vitro* procedure, described by Chadwick and Lowry (1970).

b) RADIOIMMUNOASSAY OF ACTH

(i) Standards

The present study employed Lerner Upton fraction 8B natural human ACTH for standardisation and iodination. The ACTH was prepared by Lerner and his colleagues (1968) and kindly supplied by Dr. R.W. Bates of the N.P.A., N.I.H., Bethesda, U.S.A. This has an intravenous steroidogenic potency of 101 I.U./mg $\pm$ 19 (S.D.) relative to the 2nd International Standard (Besser *et al.*, 1971). Since ACTH adsorbs to glass, especially at low hormone concentrations. but not significantly to polystyrene, all standards were prepared in polystyrene tubes and ACTH containing solutions were thoroughly equilibrated with glass pipettes before dispensing. Dried powders were initially dissolved in 0.05M phosphate buffer, pH 7.4, containing albumin (2.5 mg/ml), to give a concentration of 50 ng/ml (working standard)

The immunological potency of natural α_p^{1-39} ACTH (3rd I.S.) was 68% ($\pm$ 3, n = 19) relative to the natural α_h^{1-39} ACTH (Lerner Upton) using the N-terminal antiserum

13-18. Fresh ACTH aliquots were thawed for each assay to avoid the possibility of deterioration in the liquid state. Once frozen, the standards were discarded, as re-freezing and thawing reduced the potency of the Lerner Upton ACTH by approximately 25%. After storage at - 70° for one year, there was also approximately a 25% deterioration in potency.

(ii) Antisera

The following procedure was employed for immunisation: one mg Zinc-Synacthen (CIBA) was mixed with 500 mg Norit-X, 60 mg belloid (a detergent) and 1 ml distilled water. The mixture was thoroughly emulsified with 6 ml complete Freund's adjuvant to form a water-in-oil emulsion. Two ml of the mixture was injected into each of four random-bred New Zealand white rabbits - 0.5 ml intraperitoneally, 0.5 ml intra-dermally (neck), and 0.5 ml into each of the gluteal muscles. Four weeks after the initial injection the same dose was repeated, and the animals bled via an ear vein after a further ten days. Two of the rabbits produced antisera which bound 50% of labelled human ACTH at dilutions of 1/200 and 1/1000 on overnight incubations. The titres were not improved by further injections, and the antisera were not suitable for radioimmunoassay, because of their low titre and avidity. Several more satisfactory antisera were made available and the details of their production are shown in Table 5.

Sodium azide was added to all antisera, which were aliquoted into vials and deep frozen. Stock working solutions were made by diluting the antisera with diluent buffer (without mercaptoethanol) 100-fold, and storing at 4° for up to six months. Each was subjected to a rapid screening test which involved incubating serial dilutions

Predominant binding site on ACTH molecule.	Titres at which antisera bind 50°/o of labelled α_h ACTH (50 pg)	Raised to:	Source:
13-18 AB	1/3000 - 1/6000	Synacthen	Dr. J. Girard (Basel)
14-24 AB	1/1000 - 1/3000	Synthetic α_h ACTH	Prof. J. Landon (London)
1-24 AB	1/6000 - 1/10,000	Synacthen coupled to rabbit albumen	Dr. L. Rees & J.Kendall. (Oregon)
17-39 AB	1/5000 - 1/10,000	Natural α_h ACTH.	Burroughs-Wellcome & St. Bartholomews. (174/6)
33-39 AB	1/3000 - 1/6000	Synthetic α_h ACTH.	Burroughs-Wellcome & St. Bartholomews. (H5)

Table 5 : Details of ACTH antisera used in the present study.

with labelled ACTH under conditions approximating those eventually used in an assay (usually 18-24 hr.incubation time). The dilution of the antiserum which bound approximately 50% labelled ACTH (25-100 pg) in the absence of unlabelled ACTH was used for the assay (Table 5).

The sera were characterised by determining the ability of synthetic and natural ACTH peptides to compete with I^{125}labelled α_h^{1-39}ACTH for their binding sites. The percentage of label bound to antibody was plotted against the log.molar concentrations of the following synthetic peptides, made available by CIBA, Basel: α-MSH, α^{1-24}ACTH, α^{11-24}ACTH, $\alpha^{1-16}(NH_2)$ ACTH, α_p^{17-39}ACTH, α_p^{25-39}ACTH, $\alpha^{7-13}(NH_2)$ ACTH. Ferring, Malmo, kindly provided the following: α_h^{1-39}ACTH, α_h^{1-28}ACTH, α_h^{1-32}ACTH, and α-MSH. (The synthetic human peptides contain the unrevised sequence, and the synthetic whole peptide had low biological potency - 8.6 IU/mg). In addition to the synthetic peptides, natural porcine β-MSH was kindly provided by Dr. J.W. Kendall, Oregon. The dried powders were dissolved in 0.005N HCl at a concentration of 1 mg/ml and then diluted in diluent (without mercaptoethanol) to a final concentration of 1 μg/ml (working standards). Aliquots were stored at -70° and thawed just before use.

(iii) Iodination of ACTH

The method used for preparing and purifying labelled ACTH was that of Landon and his colleagues (1967), with minor modifications. For labelling, 2 mC high specific activity ^{125}I as sodium iodide (Amersham, Bucks) contained in 10 - 20 μl, 0.05M sodium phosphate buffer (pH 7.4), was transferred with a Hamilton syringe to a

flint glass vial, after addition of 10 μl 0.25M sodium phosphate buffer (pH 7.4) to the original container. Then, 5μg α_hACTH (Lerner Upton, fraction 8B) dissolved in 10 μl 0.005N HCl, 10 μl of Chloramine-T(5 mg/ml in 0.05M sodium phosphate buffer) and 10 μl sodium metabisulphite (10 mg/ml in 0.05M sodium phosphate buffer) were added rapidly in turn, with mixing for a few seconds after each addition. The complete mixture, including the flint glass tube, was immersed immediately in 20 ml 0.05M sodium phosphate buffer (pH 7.4), containing 2.5 mg/ml crystalline human serum albumin (Lister Institute) in a 50 ml ground glass stoppered centrifuge tube.

Purification of the labelled ACTH was performed by transferring 10ml to a polystyrene test tube (Henleys Medical Supplies, London, N6-CM 5 tubes and CM 6 caps) containing 10 mg QUSO glass (G 32 Philadelphia Quartz Co., Philadelphia, Pa.) when, after rotation for 30 minutes, maximum adsorption of undamaged labelled ACTH had occurred. After centrifugation, the supernatant was aspirated and discarded and the QUSO washed with 2 ml deionised water, centrifuged and aspirated as above. Labelled ACTH was then eluted with 2 ml of 1% acetic acid - 40% acetone-water mixture by rotation, centrifugation and careful removal of the supernatant. The label was stored at 4° and assessed for purity by chromatoelectrophoresis.

The reliability of the iodination and purification procedure was shown by the results of 50 iodinations of natural human ACTH with ^{125}I over an 18 month period. The mean yield was 73 ± 17.6% (S.D.) with 8.6 ± 3% being 'damaged'. After purification, the mean 'maximum binding' (percent label bound to a 1/500 dilution of 33-39 AB after

1 hour incubation) was 64$\pm$ 8.9% with a 'label blank' (the percentage of label not adsorbed onto charcoal in the absence of antibody) of 7.8 $\pm$ 3.1. The mean specific activity achieved was no greater than 500 µC/µg (c. one iodine molecule per ACTH molecule). Labelled ACTH retained satisfactory immunoactivity for two weeks.

(iv) Separation Method

Antibody bound and free ACTH were separated by addition of 200 µl of a charcoal suspension containing Norit-X activated charcoal, 0.85 g Dextran T-70, 10 ml 0.5M sodium phosphate buffer (pH 7.4) and 60 ml horse serum (Natural clot No.3, Burroughs Wellcome) diluted with deionised water to 100 ml, (Landon and Greenwood, 1968). All tubes were thoroughly mixed and centrifuged. The supernatant was aspirated and the charcoal pellet, to which the free ACTH was adsorbed, counted. Solutions were only used for three days after being made.

(v) Assay conditions

Serial dilutions of an extract or peptide were compared with similar dilutions of an appropriate concentration of standard ACTH. 10-25 µl of antiserum. to give an appropriate final dilution (see above), and 10 µl of labelled ACTH containing 25 - 100 pg ACTH were added to each tube and the total incubation volume was 220 µl. One set of tubes, contained 200 µl diluent and labelled ACTH only ('Label blanks') and another, labelled ACTH plus antibody ('antibody blanks').

The tubes were mixed and incubated overnight at 4°, then counted in an automatic gamma well-counter (Wallac) till at least 5000 counts were generated. Antibody-bound and free ACTH were then separated as described and the

charcoal pellet was counted. The percent of labelled ACTH bound to antibody was calculated, the standard curve plotted, and the concentration of ACTH in the unknown sample was calculated from the middle portion of the sigmoid curve.

Typical standard curves for the five antisera employed in this study are shown in Fig. 3.

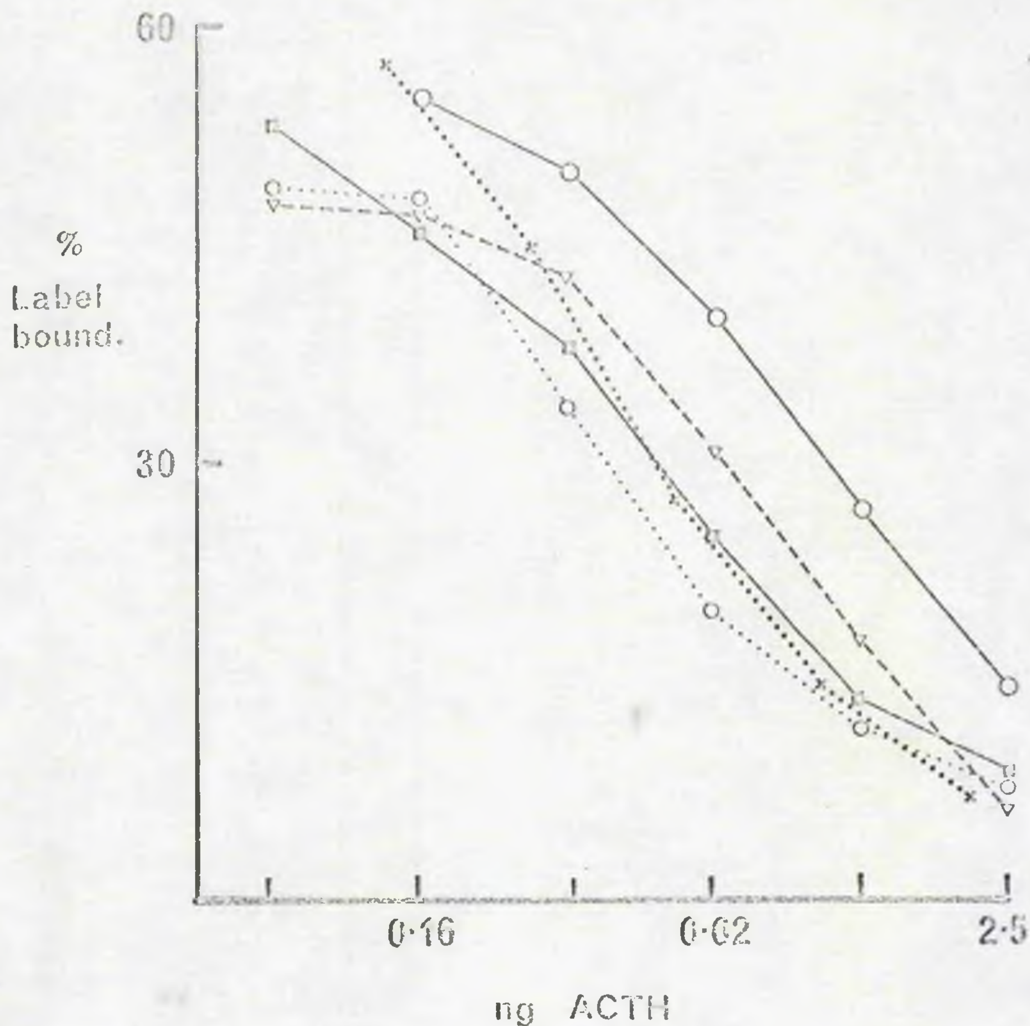


Figure 3 : Typical 'standard curves' given by the five antisera used in the present study. Natural human ACTH, prepared by Lerner and Upton , was used for standardisation and iodination.

□———□ , 13–18 AB

▽------▽ , 14–24 AB

○·······○ , 17–39 AB

○———○ , 33–39 AB

× ·······× , 1–24 AB

IMMUNOHISTOLOGICAL AND ORGAN CULTURE PROCEDURES

SECTION 3

a) IMMUNOFLUORESCENT HISTOLOGY

The immunofluorescent procedure employed was as follows:- (Ref. Nairn, 1969).

1) Freshly dissected rat pituitaries were placed in Tissue-tek O.C.T. Compound (Ames, Indiana), and rapidly frozen in a dry ice/iso-pentane mixture.

2) Ten µm sections were cut in a cryostat, mounted on glass slides, fixed in 70% ethanol for 30 minutes, and rinsed in veronal-HCl buffer (pH 7).

3) The test antiserum (in dilutions ranging from 1/4 to 1/32) was placed over the sections, and left for 20 minutes.

4) Slides were rinsed with buffer for 10 minutes.

5) Goat anti-rabbit gamma globulin labelled with fluorescein (Behringwerke AG. Hoechst OTKF 05) was placed over the sections (1/20 dilution) and left for 20 minutes.

6) Slides were rinsed with buffer for a further 20 minutes.

7) Sections were mounted in glycerine, with a cover slip and observed with an ultra-violet light source (490 nm) on a Laborlux microscope.

b) ORGAN CULTURE

Rat anterior and posterior pituitary lobes were maintained in non-proliferative organ culture (Trowell, 1959). Each lobe was placed on defatted lens paper, on a stainless steel grid, in a plastic organ culture dish (Falcon). Waymouth's medium (1959) enriched with 20% calf serum was added to the level of the grid (approximately 1.5 ml) and

the dishes placed in an incubator at 37° in an atmosphere of 5% CO_2/95% O_2. Media were changed at intervals, transferred to plastic storage tubes and frozen immediately. At the end of the experiments, anterior and posterior lobes were also frozen, prior to extraction with dilute hydrochloric acid.

RESULTS AND PRELIMINARY DISCUSSION

SECTION 1

CHARACTERISATION OF METHODS

a) RADIOIMMUNOASSAY

(i) Specificity of antisera The predominant binding sites of the five antisera used in this study were determined as described by Orth and his colleagues (1968). Four types of inhibition curve were found:

1) A curve parallel to that given by the full peptide, and of an equivalent potency on a molar basis (e.g. cross-reaction of N-terminal antiserum with α^{1-32}ACTH. Fig.4). It is assumed, in this situation, that the peptide fragment contains the full antigenic determinant.

2) A curve parallel to that given by the full peptide, but of considerably less potency (e.g. cross-reaction of N-terminal antiserum with $\alpha^{1-16}NH_2$ACTH, Fig.4). Based upon data obtained by Byfield (1972) using calcitonin fragments (in which he showed that progressive removal of NH_2-terminal amino acids from a peptide, in which the antigenic determinant lay at the NH_2-terminus, resulted in a progressive fall in potency in the radioimmunoassay, without altering the slope of the inhibition curve) this suggests that the peptide contains only a part of the antigenic determinant.

3) A curve parallel to that given by the standard at low concentrations of the peptide, but lying horizontal at high concentrations (e.g. cross-reaction of C-terminal antiserum with α^{1-24}ACTH and α^{1-28}ACTH, Fig. 5). It is assumed, in this situation, that the antiserum contains two or more populations of antibodies directed against different determinants, and that the peptide fragment

being tested contains only one of these determinants.

4) A horizontal curve, indicating failure of the peptide to displace labelled ACTH (e.g. cross-reaction of N- and C- terminal antisera with α-MSH and β-MSH, Figs. 4 and 5). It is assumed in this situation that the peptide does not contain the antigenic determinant(s).

The availability of several synthetic ACTH peptides allowed the selection, in the present study, of five antisera with binding sites on different parts of the ACTH molecule. Two of these proved particularly useful in that they reacted specifically with determinants within and outside the biologically active portion of the mammalian ACTH molecule, and were the only two antisera that gave parallel displacement curves with a wide range of vertebrate pituitary extracts (thus allowing quantitation). The antiserum directed towards the biologically active portion of the molecule was termed 'N-terminal' and the data shown in Figure 4 indicate that its antigenic determinant lies in the region between the 13th and 18th amino acids of ACTH (13-18AB). The antiserum directed against the non-biologically active portion of the mammalian ACTH molecule was termed 'C-terminal' and the data shown in Figure 5 indicate that two determinants exist - one within the 17 to 24 portion and the other within the 33 to 39 portion of the mammalian ACTH molecule (33-39 AB).

The three other antisera studied were predominantly directed at sequences within the 14 to 24 (14-24 AB), 1 to 24 (1-24 AB) and 17 to 39 (17-39 AB) portions of the mammalian ACTH molecule. 14-24 AB had an identical specificity to the N-terminal antiserum except for a

strong, but incomplete cross-reaction with the α_p^{17-39} ACTH fragment (type 3 curve). The 1-24 AB cross-reacted with all peptides containing a part of the 1 to 24 region, although full cross-reaction required the presence of the whole of this portion of the ACTH molecule. The 17-39 AB cross-reacted with a large range of fragments containing part or whole of the 17 to 39 portion of mammalian ACTH and its specificity has been published (Ratcliffe et al., 1972).

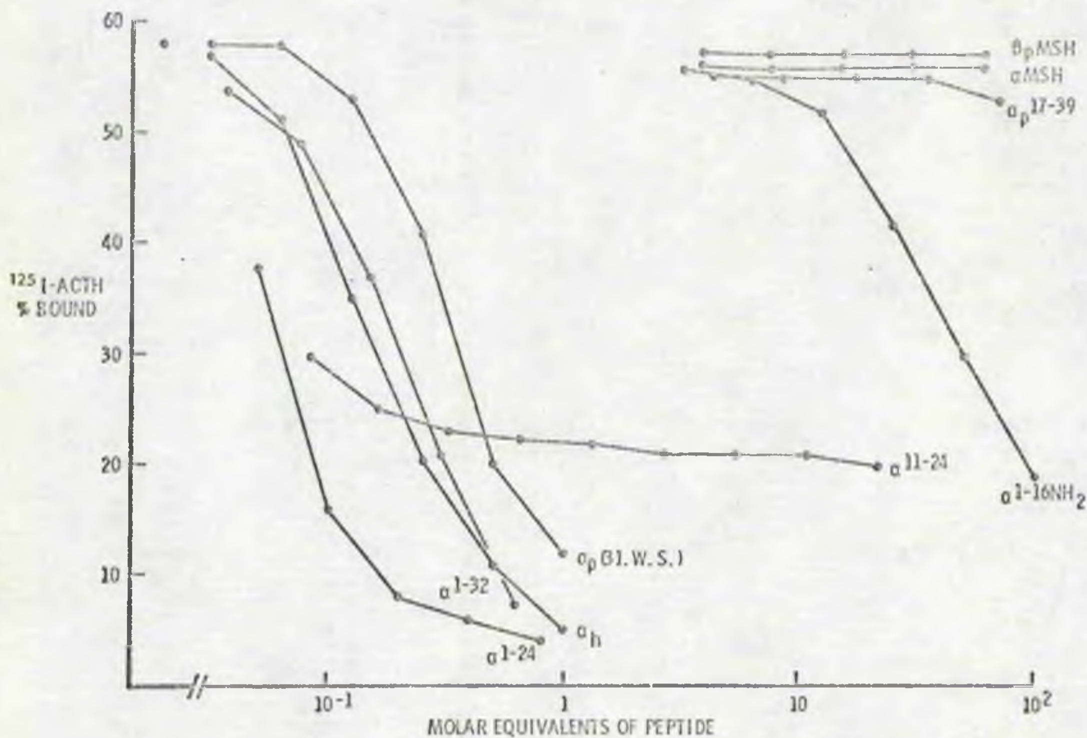


Figure 4 : Specificity of N-terminal antiserum (13-18 AB) .

Full cross-reaction is given by α^{1-24}ACTH and other molecules containing the intact α^{1-24} sequence (α_h^{1-32} - , α_h - , α_p -ACTH). Since the antiserum reacts less than 1% with $\alpha^{1-16}NH_2$ ACTH , α-MSH , β_p-MSH and α_p^{17-39}ACTH the antigenic determinant may bridge the 16 and 17 positions. The incomplete cross-reaction of α^{11-24}ACTH indicates that peptide sequences outside this region may be important in maintaining conformation of the antigenic determinant . The conditions of incubation were 1/3000 final dilution of antiserum, 100 pg of ^{125}I -α_h ACTH , and overnight incubation. (Oxytocin, AVP , LVP and bovine neurophysin in μg quantities failed to displace labelled ACTH).

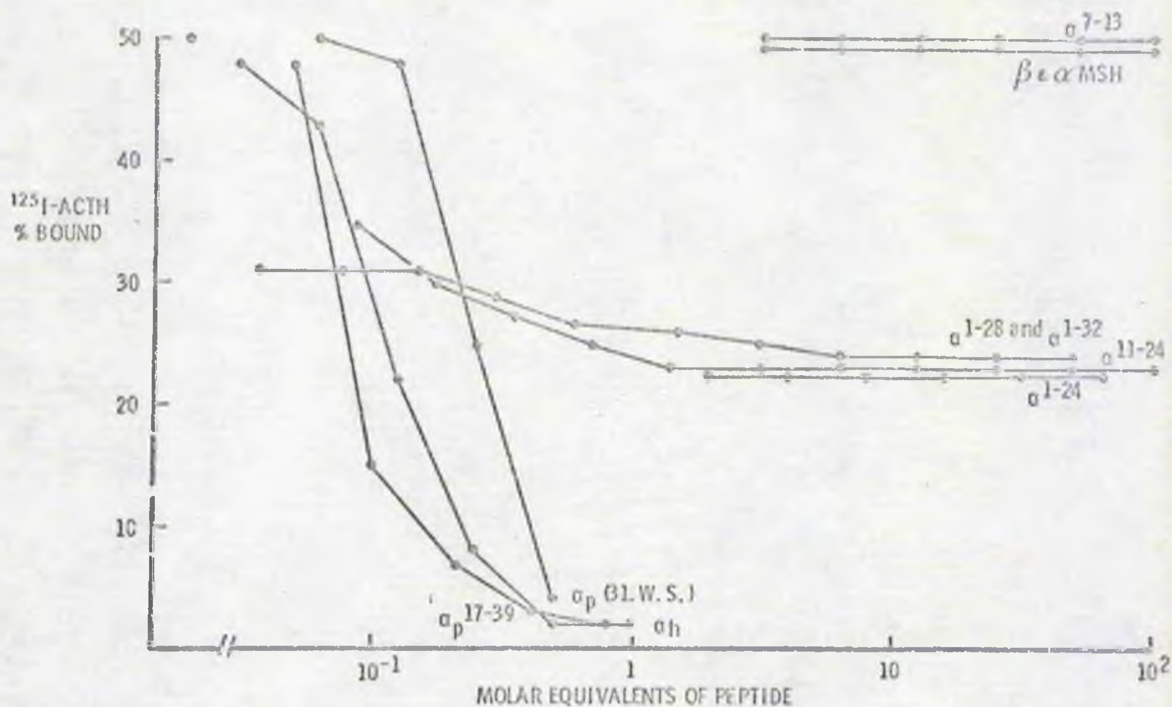


Figure 5 : Specificity of C-terminal antiserum (33-39 AB).

Full cross-reaction is given by α_p^{17-39}ACTH and other molecules containing the intact α^{33-39}sequence (α_h - , α_p - ACTH). Incomplete and non-parallel cross-reactions are given by α^{1-24}ACTH , α_h^{1-32}ACTH and α_h^{1-28}ACTH so that such peptides can be distinguished from those containing the full antigenic determinant. The conditions of incubation were the same as those for the N-terminal antiserum. (Oxytocin, AVP , LVP and bovine neurophysin in μg quantities failed to displace label).

b) EXTRACTION

(i) Validation of rapid extraction procedure

Nine rat anterior pituitary lobes were divided into approximately equal halves, which were weighed, extracted separately and assayed with the N-terminal antiserum. The mean ACTH concentrations in left and right halves of the anterior lobes were respectively 143.5 (± 41.6 S.D.) and 148.6 (± 39.9 S.D.) μg/g wet weight tissue. There was no statistically significant difference between these groups, indicating that the extraction method was reproducible. Additionally, N-terminally immunoactive ACTH was shown to be stable in dilute hydrochloric acid extracts for up to 24 hours at room temperature, and for at least eight days at -70°.

(ii) Extraction of ACTH-like peptides from acetone-dried powder

The requirements for the large scale extraction procedures were that (1) there should be maximal yield, (2) there should be partial purification and (3) the extracted peptides should be stable. Several extraction procedures were tested, using porcine posterior pituitary lobe powder. The extracts were assayed with both N- and C-terminal antisera and the yields, purity and stability compared.

1) Yield:

One g portions of powder were weighed out and extracted by the various procedures described in the methods section. After centrifugation, the supernatants were assayed for ACTH and the results are given in Table 6 (1). The total amounts of extractable N-terminally immunoactive ACTH ranged from 0.3 mg with 15% trifluoro-

acetic acid to 2 mg with 5% acetic acid (boiled), and of C-terminally immunoactive ACTH from 0.8 mg with 5% acetic acid to 4.5 mg with hydrochloric acid (boiled). C/N ratios ranged from 1.0 (5% acetic acid) to 7.8 (0.1N hydrochloric acid). Re-extraction of the dried residues with 0.02N hydrochloric acid revealed amounts of ACTH ranging from undetectable in the 5% acetic residue (not boiled) to 3.4 mg C-terminal activity in the 15% trifluoroacetic acid residue. In most cases the amounts of activity left in the residues - Table 6 (2) - were inversely proportional to those that had been extracted, but in the case of the 5% acetic acid extraction the yield of C-terminally immunoactive ACTH and the amounts of activity left in the residue were both low (see below).

The total yield of ACTH in the powdered extracts was assessed by assay of portions re-extracted with 0.02N hydrochloric acid-Table 6 (3). The yields of N-terminally immunoactive ACTH ranged from 0.07 mg with 15% trifluoroacetic acid to 0.6 mg with acetic acid, and C-terminally immunoactive ACTH yields ranged from 0.7 mg with 15% trifluoroacetic acid to 2.8 mg with glacial acetic acid.

2) Purity:

Although the total yields of ACTH by several methods were comparable, only three procedures gave powders in which activity was greater in terms of micrograms per gram of powder than in the original crude acetone-dried powder. These were glacial acetic acid, HCl/acetone and TFA/acetone (Fig. 6).

3) Stability:

A loss of C-terminally immunoactive ACTH in the 5% acetic acid extract was noted in the previous experiments. The presence of proteolytic enzyme activity could be inferred from the failure of similar degradation to occur in the heated 5% acetic acid extract, or in extracts with stronger acids. Further evidence for this was obtained by mixing one g of acetone-dried posterior pituitary powder with 20 ml 5% acetic acid at room temperature and, at timed intervals, removing aliquots which were boiled, centrifuged and assayed (Fig. 7). After one hour, C-terminally immunoactive ACTH had fallen from 4 to 0.93 μg per mg powder, while N-terminally immunoactive ACTH remained unchanged for up to 24 hours.

In a further experiment, portions of posterior pituitary lobe powder were mixed with a range of sodium acetate buffers for 90 min at 20°, and assayed with both antisera. C-terminally immunoactive ACTH concentrations fell only at pH 4, indicating that the enzyme had a narrow pH range.

The extraction experiments demonstrated that the highest yields of C-terminally immunoactive ACTH from acetone dried powder were obtained by using trifluoroacetic acid or hydrochloric acid/acetone mixture or glacial acetic acid. They also demonstrated that addition of aqueous acetic acid to the powder was associated with the disappearance of C-terminal activity, which could be counteracted by boiling or use of stronger acids.

Pickup and Hope (1971) have previously demonstrated proteolytic activity in bovine posterior pituitary lobe powder at pH 3.8.

PROCEDURE	(1) SUPERNATANTS						(2) RESIDUES						(3) POWDERED EXTRACTS					
	Wt. g.	N µg/mg	C µg/mg	N mg	C mg	C/N ratio	Wt. g.	N µg/mg	C µg/mg	N mg	C mg	C/N ratio	Wt. g.	N µg/mg	C µg/mg	N mg	C mg	C/N ratio
0.1 N HCl	1	0.5	3.9	0.5	3.9	7.8	0.25	1.0	6.3	0.3	1.6	5.3	0.37	1.1	5.0	0.4	1.9	4.8
0.1 N HCl/ BOILED.	1	0.6	4.5	0.6	4.5	7.5	0.33	0.5	8.9	0.2	2.9	15.0	0.29	0.7	5.4	0.2	1.6	8.0
15 °/o TFA	1	0.3	1.3	0.3	1.3	4.3	0.19	1.0	7.0	0.5	3.4	7.4	0.57	0.2	1.6	0.1	0.7	8.0
5 °/o HAc	1	0.8	0.8	0.8	0.8	1.0	0.18	0.2	0.4	0.02	0.1	-	0.38	1.7	2.0	0.6	0.8	1.3
5 °/o HAc/ BOILED .	1	2.0	4.4	2.0	4.4	2.2	0.34	0.5	2.3	0.2	0.8	4.6	0.39	1.5	5.0	0.6	1.9	3.3
GLACIAL HAc.	1	1.4	4.1	1.4	4.1	3.0	0.69	0.5	1.4	0.3	1.0	3.0	0.18	3.2	15.5	0.6	2.8	4.7
HCl/ACETONE	1	-	-	-	-	-	0.62	0.2	0.6	0.1	0.4	3.5	0.06	4.0	10.4	0.2	0.6	2.6
TFA/ACETONE	1	-	-	-	-	-	0.66	0.1	0.8	0.1	0.5	7.0	0.12	4.0	17.5	0.5	2.0	4.3

Table 6 : ACTH content of porcine posterior pituitary lobe powder as determined by several extraction methods. The extracts were assayed before freeze-drying (1). Portions of the freeze-dried extracts, or powdered precipitate from HCl/acetone and TFA/acetone extractions were re-extracted in 0.1N HCl and assayed(3). Residues were re-extracted in the same fashion and assayed to determine the amount of activity remaining (2).
N - immunoreactive ACTH measured with the 13-18 AB ; C - immunoreactive ACTH measured with the 33-39 AB ; µg/mg - µg ACTH per mg powder ; mg - mg ACTH (total yield).

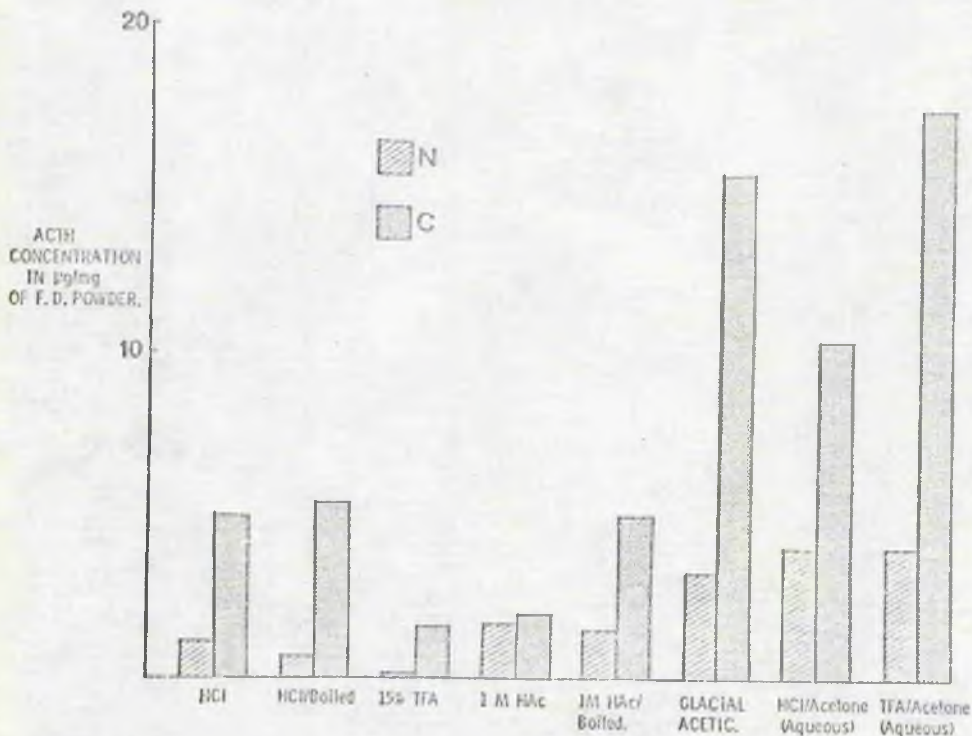


Figure 6: Concentration of ACTH in powdered extracts of porcine posterior pituitary lobe powder. Extracts were re-extracted in 0.1N hydrochloric acid and radioimmunoassayed with the N-terminal (13-18 AB - hatched blocks) and C-terminal (33-39 AB - shaded blocks) antisera. The ACTH concentration in the extracts is shown on the ordinate in µg ACTH per mg powder. The figure demonstrates the partial purification of ACTH achieved by extraction with glacial acetic acid, HCl/acetone and TFA/acetone.

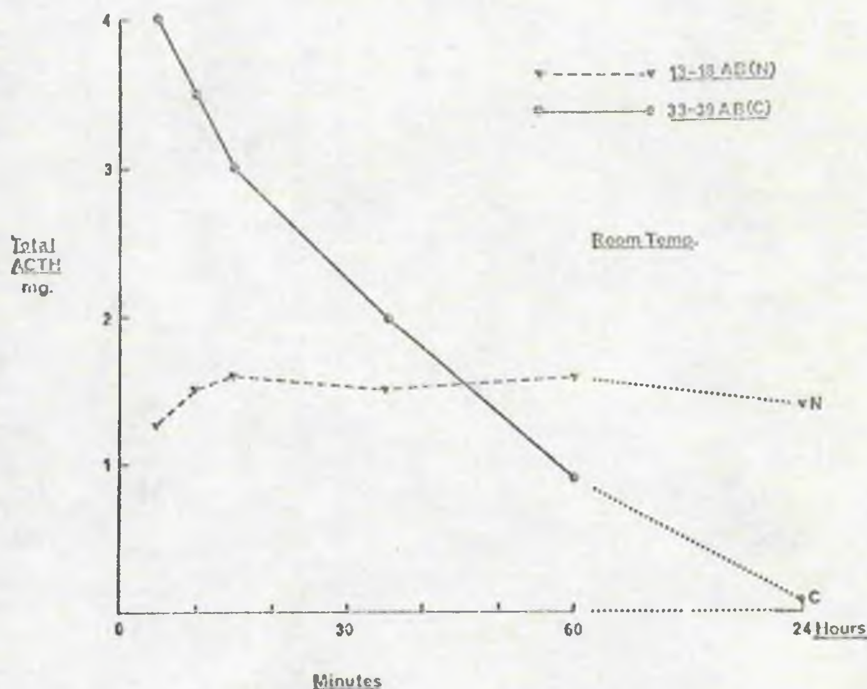


Figure 7: Immunoactive ACTH content of 5°/₀ acetic acid extract of 1 gram porcine posterior pituitary lobe powder. Aliquots were taken from the extract at timed intervals after addition of the acetic acid to the powder and boiled, centrifuged and assayed with the N-terminal (13-18 AB) and C-terminal (33-39 AB) antisera. This study demonstrates the rapid disappearance of C-terminal activity in 5°/₀ acetic acid extracts of the powder.

SECTION 2

COMPARATIVE STUDIES

a) IMMUNOLOGICAL CHARACTERISATION OF VERTEBRATE CORTICOTROPHINS

Doubling dilutions of pituitary extracts of several species were assayed using 13-18 AB, 14-24 AB, 17-39 AB and 33-39 AB, and the results are displayed diagramatically (Figs 9 and 10). Figure 8 shows the cross-reaction of a human pituitary extract with the four antisera. For clarity and ease of comparison, the binding of label in the absence of added unlabelled ACTH has been termed 100%.

(i) Radioimmunoassay with 13-18 AB Each pituitary extract contained material which gave parallel displacement over part or all of the curve as compared to the ACTH standard, except for that from the lamprey and from dogfish rostral and neurointermediate lobes. It was concluded, therefore, that pituitaries of all species studied (except those of the lamprey and dogfish) contained a peptide immunologically similar to human α^{13-18} ACTH. The content of active material in the lamprey pituitary was so small that the apparent displacement of labelled ACTH could be artefactual. In the dogfish, the non-parallel displacement curves given by the pituitary extracts suggest that its corticotrophin possesses a slightly different α^{13-18} sequence to that of mammalian ACTH.

(ii) Radioimmunoassay with 14-24 AB. Dogfish, powan, gecko, egret and rat pituitary extracts contained material which yielded non-parallel displacement curves that were less steep than those of the ACTH standard. It was

concluded, therefore, that their pituitary glands contain a cross-reacting material that differs from the reference ACTH in the portion α^{14-24}ACTH. Cod and toad pituitary extracts failed to cause any significant displacement of the labelled ACTH, and it was concluded that the α^{14-24} portion of the molecule differs considerably from human ACTH in these species. This antiserum is not useful for quantitative assessment of immunoactive ACTH in species other than man, because of the lack of parallelism, but it does highlight species differences.

(iii) Radioimmunoassay with 17-39 AB. The pituitary extracts of all the non-mammalian vertebrates studied reacted in the same way with 17-39 AB as with 14-24 AB. It was concluded that corticotrophins of these species differed structurally in the C-terminal portion of the molecule from that of the α^{17-39}portion of human ACTH. Rat anterior pituitary extracts, however, gave full displacement curves parallel to the standard, indicating that rat ACTH is probably similar to human ACTH in this portion of the molecule.

(iv) Radioimmunoassay with 33-39 AB. Pituitary extracts from fish and amphibians caused only slight displacement of labelled ACTH from 33-39AB. In the remaining species, however, pituitary extracts appear to contain material that is immunologically indistinguishable from the C-terminal portion of human ACTH. Competition curves for all species studied with this antiserum are shown in Fig.11.

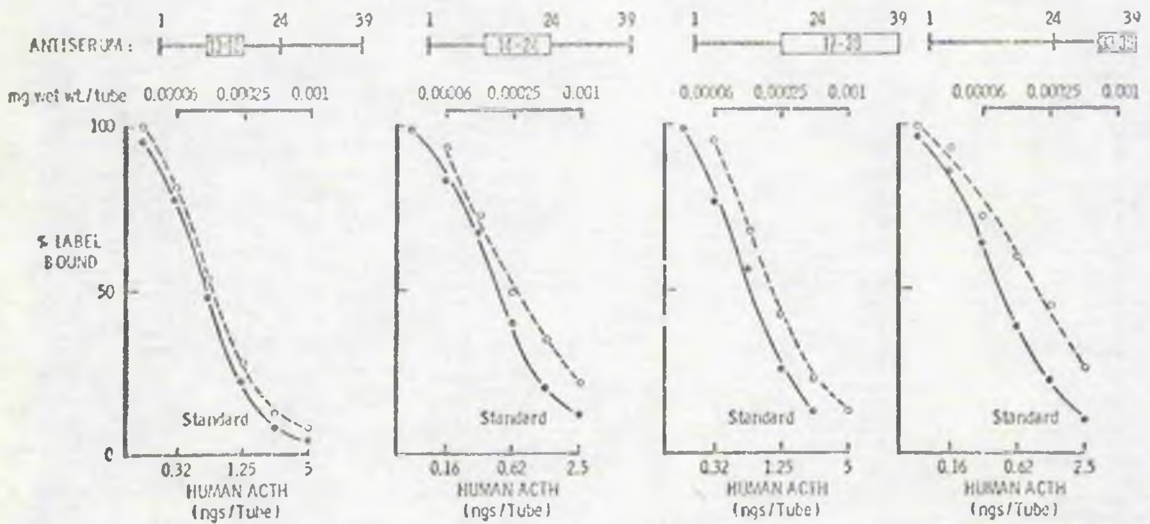


Figure 8: Competitive binding curves given by a human pituitary extract with 1–13 AB , 14–24 AB , 17–39 AB and 33–39 AB . Antibody bound labelled human ACTH is plotted on the ordinate. The scale is expanded so that the proportion of label bound by antibody in the absence of unlabelled ACTH is equivalent to 100°/₀. The amounts of reference standard (solid line) and of pituitary tissue (broken line) are plotted on a logarithmic scale as ngs of hormone and mgs of wet tissue respectively.

13-18 AB   14-24 AB   17-39 AB   33-39 AB

LAMPREY

DOGFISH

POWAN

COD

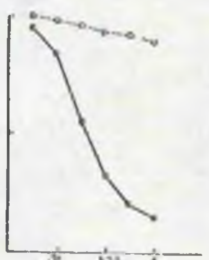


Figure 9: Competitive binding curves given by lamprey, dogfish, powan and cod pituitary extracts in the ACTH radioimmunoassay with 13-18 AB, 14-24 AB, 17-39 AB and 33-39 AB. The method of plotting the curves is described in Figure 8. The tissue concentrations have been omitted for simplicity.

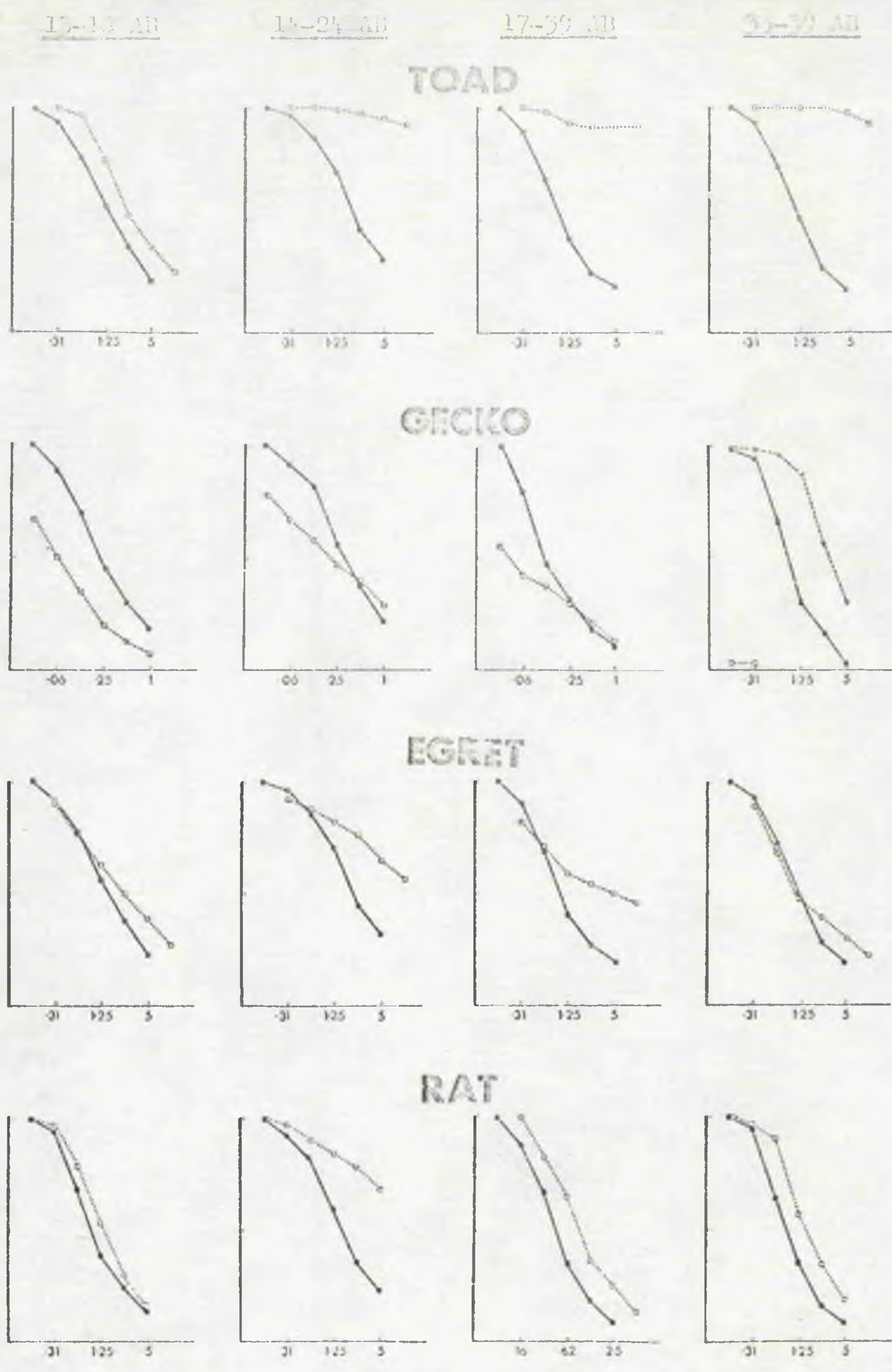


Figure 10 : Competitive binding curves given by toad, gecko, egret and rat pituitary extracts in the ACTH radioimmunoassay with 15-18 AB, 14-24 AB, 17-39 AB and 33-39 AB. The method of plotting the curves is described in Figure 8. The tissue concentrations have been omitted for simplicity.

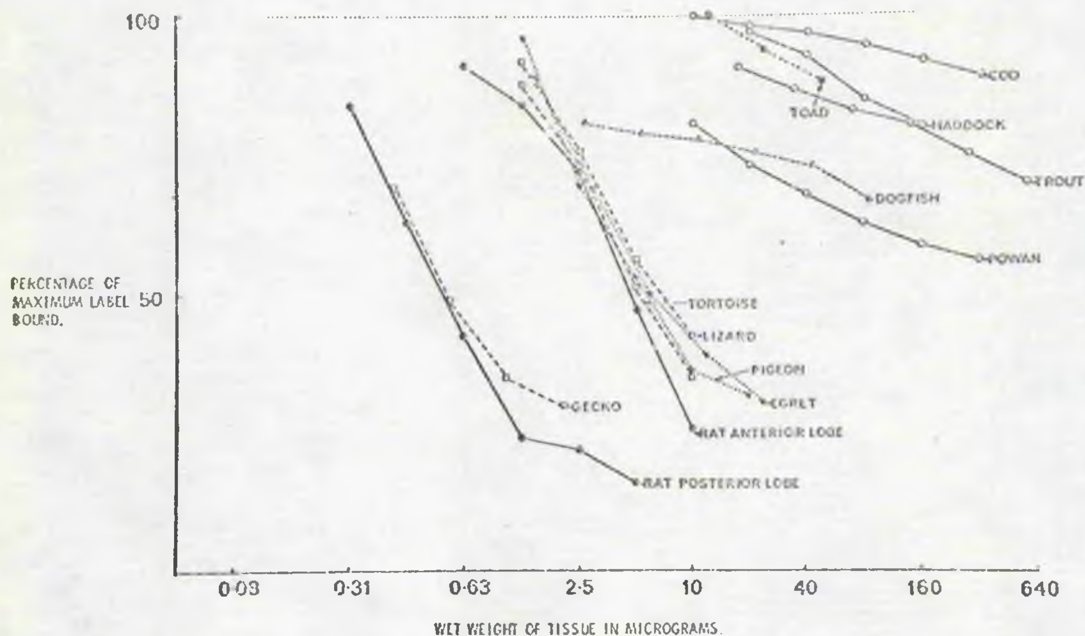


Figure 11: Competitive binding curves of vertebrate pituitary extracts in the ACTH radioimmunoassay using the C-terminal antiserum, 33-39 AB. The percentage of labelled ACTH bound to antibody is shown on the ordinate - the scale has been expanded to 100% to allow a comparison of the curves , which were obtained at different times. The wet weight of tissue extracted is shown in micrograms.

b) PITUITARY CONTENT OF CORTICOTROPHIN IN SEVERAL VERTEBRATE SPECIES

A quantitative assessment of corticotrophin concentrations was made in several vertebrate species by radioimmunoassay using both 13-18 AB and 33-39 AB, and by bioassay (Table 7). Whole pituitaries or separate pituitary lobes were extracted singly or in batches. Bioassay and N-terminally immunoactive ACTH levels (13-18 AB) were calculable in every case, although displacement curves with dogfish and lamprey extracts were non-parallel to those of the ACTH standard, so that only approximate values can be given. The limits of detection of the C-terminal radioimmunoassay are quoted alongside the N-terminal values for those animals in which pituitary extracts failed to cross-react (fish and amphibians).

(i) Bioassay of ACTH: A wide variation in pituitary corticotrophic activity was observed in vertebrate pituitaries, ranging from picogram amount in lampreys to microgram amounts in several mammals. Detailed studies were precluded by the nature of the assay, which was both costly and time consuming.

(ii) Radioimmunoassay with N-terminal antiserum:

Immunoactive ACTH concentrations determined with the 13-18 AB were generally higher, but showed as wide a variation as the bioassay results.

(iii) Radioimmunoassay with C-terminal antiserum:

In the amphibians and fish, an absence of cross-reaction with the 33-39 AB was manifested by C/N ratios of less than unity whereas, in the higher vertebrates, C-terminal activity often exceeded N-terminal activity.

Total amounts of activity ranged from 6 ng in lizards to 21 µg in a guinea-pig. The apparent absence of C-terminal activity in pig and human pituitaries is considered below.

(iv) ACTH concentrations in relation to body weight in several vertebrates:

N-terminally immunoactive corticotrophin levels were related to body weight (ng ACTH/g body weight) with the following results: lamprey, 0.0017; cod, 0.26; powan, 0.24; rat, 4.0; pig, 4.1; man, 3.1. These figures demonstrate the low content of ACTH in lamprey pituitaries.

(v) Further study on lamprey pituitaries.

Dissected pro- and meta- plus meso-adenohypophyses from ten lampreys were grouped into two batches, which were extracted separately in 0.1 N hydrochloric acid and then assayed at greater sensitivity than usual with the 13-18 AB . There was no detectable activity (limit of detection = 200 pg/lobe) in any of the extracts. Whereas 12% of a lobe failed to displace label from the 13-18 AB, a dilution representing 1/256 of a lobe displaced label from 1-24 AB (Fig. 12a). This antiserum cross-reacts with both mammalian and dogfish α-MSH (see below), and it is evident that the levels of immunoactive material are higher in posterior lobe than in anterior lobe extracts.

(vi) Studies on dogfish α-MSH.

Dogfish α-MSH (kindly supplied by Dr. Lowry) was assayed with the 13-18 AB (0.3 ng/µg), with the 1-24 AB (Fig.12b), and by bioassay - (0.25 ng/µg single point assay). The displacement curves given by the purified α-MSH with the 1-24 AB were non-parallel to those of the human standard, but paralleled displacement curves given by

neuro-intermediate lobe extracts (cf. lamprey).

(vii) Studies on human and pig pituitaries.

The low levels of C-terminal activity in human and pig pituitaries (Table 7) were due to proteolytic degradation of this portion of the molecule in the initial crude extracts, due to the use of insufficient 0.02N HCl to maintain a low pH in the presence of large quantities of tissue (4 mls 0.02N HCl/three pituitaries). The final pH in the extracts was 4 (see p. 62).

Table 7 : Pituitary content of corticotrophin in several vertebrate species.

Notes : -

1) Biological activity measured by the in vivo Lipscomb-Nelson assay.

2) N-terminal immunoactivity - ACTH content measured with the 13-18 AB, using human ACTH for standardisation and iodination . (In the case of lamprey and dogfish pituitary extracts competitive binding curves were not parallel to those given by the ACTH standard,but approximate values are given.)

3) C-terminal immunoactivity - ACTH content measured with the 33-39 AB, using human ACTH for standardisation and iodination.

4) Dogfish neurointermediate lobes were not separated from the saccus vasculosus tissue.

5) Figures which are underlined represent the mean of several estimations (denoted by'No.') on separate extracts. In all other situations, any figure greater than 1 in the 'No.' column represents a pool of pituitaries.

	No.	Wet wt. mg.	Biological activity conc.	Biological activity total	N-terminal immunoact. conc.	N-terminal immunoact. total	C-terminal immunoact. conc.	C-terminal immunoact. total	C/N Ratio
1) CYCLOSTOMES			ng/mg	ng	ng/mg	ng	ng/mg	ng	
Lampetra fluviatilis Whole pituitary	25	-	-	.06	-	.09	-	-	-
Pro-adeno-hypophysis	5	-	-	-	-	.2	-	-	-
Meta-adeno-hypophysis	5	-	-	-	-	.2	-	-	-
2) ELASMOBRANCHS									
Squalus acanthias Rostral lobe	1	3.5	140	500	488	1500	5.2	18	.01
	1	8.7	-	-	471	4158	-	-	-
	1	10.9	-	-	604	6584	-	-	-
	-	25.5	204	-	573	-	-	-	-
	-	43.8	-	-	440	-	5	-	.01
	-	21.9	-	-	340	-	7	-	.02
	-	16.0	-	-	1250	-	7	-	.01
Neuro-intermediate lobe (note 4)	1	37.7	-	-	3.1	112	-	-	-
	1	41.8	0.9	38	3.9	163	-	-	-
	1	36.4	0.2	7	2.2	80	-	-	-
	-	100	-	-	2.5	-	1.6	-	.06
	-	120	-	-	3.3	-	1.3	-	.04
3) TELEOSTS									
Coregonus clupeoides (Loch Lomond powan)	15	31	12.5	25	13.5	27	1.4	2.8	.10
	1	2.0	11.1	22	27.0	54	-	-	-
	25	43.3	2.0	3	8.8	15	-	-	-
	20	43.4	1.2	3	7.1	16	-	-	-
	17	20.5	-	-	19.3	23	3	3.6	.15
	15	2.2	-	-	20.2	44	-	-	(note 5)
Gadus morhua (arctic cod)	1	15.5	5.2	81	55	853	1.2	19	.02
	1	12.8	-	-	32	410	0.5	6	.02
	-	118	-	-	17	-	1.8	-	.10
	1	10.4	-	-	24	250	-	-	-
	1	12.3	-	-	15	185	-	-	-
	1	14.2	-	-	17	241	-	-	-
	1	20.6	-	-	13	268	-	-	-
Clupea harengus (herring)	20	38.3	17.0	32	11	21	1.7	3.2	.20
Melanogrammus aeglifinus (haddock)	5	25.7	-	-	21	107	0.8	4	.04
	3	14.5	-	-	13	62	0.8	3	.06
Salmo trutta (trout)	1	4.2	-	-	15	63	1.5	6	.10
4) AMPHIBIANS									
Bufo bufo (toad)	2	2.1	93	98	148	156	12	12	.08
	2	1.9	124	121	485	473	12	12	.02
	2	0.5	-	-	1300	318	25	6	.02
Anterior lobe	3	1.0	250	80	1182	378	25	8	.02
Posterior lobe	3	0.5	10	2	61	10	10	2	.6
Salamandra salamandra (salamander)	1	0.5	-	-	4	2	5	3	1.2
	1	0.6	-	-	9	5	5	3	.6
	1	0.7	9.9	7	13	9	5	3	.4

(CONTD)	No.	wt.	Bioactivity.		N-termin.		C-termin.		
5) REPTILES									
Tarentola sp.	1	-	-	-	-	60	-	-	-
(gecko)	1	-	-	-	-	49	-	-	-
	1	-	-	-	-	33	-	-	-
	1	-	-	8	-	34	-	1000	30
	1	-	-	-	-	50	-	400	8
	1	-	-	-	-	21	-	85	4
Lacerta viridis (green lizard)	3	-	-	2	-	2.5	-	6	2.4
Testudo graeca (tortoise)	1	4.1	133	545	70	287	291	1193	4.1
6) BIRDS									
Columber columber	1	4.0	130	520	143	572	254	1016	1.8
(pigeon)	1	2.6	199	517	267	694	455	1183	1.7
	1	2.5	-	-	51	128	88	220	1.7
Mesophoyx intermedius (egret)	1	9.9	67	663	105	1040	110	1089	1.1
7) MAMMALS									
Rattus albino	5	6.7	-	-	162	1004	-	-	- (note 5)
	1	7.1	66	470	169	1200	720	5112	5
Anterior lobe	18	5.3	-	-	150	795	-	-	- (note 5)
	1	9.7	22	213	54	524	66	640	1.2
	1	5.6	29	162	78	437	91	510	1.2
	1	3.3	56	185	168	554	123	406	0.7
	1	5.9	46	271	133	785	108	637	0.8
	1	4.0	99	396	176	704	142	568	0.8
	1	9.7	-	-	65	631	46	446	0.7
	1	8.0	-	-	71	568	92	736	1.2
	1	9.8	-	-	76	745	92	902	1.2
	1	5.3	-	-	130	689	127	673	1.0
Posterior lobe	1	0.5	18	9	80	40	4634	2317	58
	1	1.3	14	18	50	65	1532	1992	31
	1	1.2	25	28	94	113	1689	2027	18
	1	1.3	-	-	30	38	852	1022	31
	1	0.9	-	-	92	83	2036	1832	23
Caveus porcellus (guinea pig)									
Anterior lobe	1	10.7	121	1295	238	2547	392	4194	1.6
	1	11.0	-	-	324	3564	612	6732	1.9
	1	11.2	-	-	185	2072	341	3819	1.9
	1	11.5	-	-	434	4991	1112	12788	2.6
Posterior lobe	1	3.7	4.0	13	20	70	452	1672	23
	1	3.0			20	60	1721	5163	86
	1	3.3	4.6	15	20	66	837	2762	42
	1	3.6			37	133	2316	8338	70
		Gram				µg		µg	
Sus scrofa (pig)	3	0.90	-	-	1541	462	80	24	.05
	3	0.74	-	-	1652	413	80	20	.05
	3	0.97	-	-	1033	331	80	26	.08
Homo sapiens (human)	3	1.98	-	-	372	246	70	46	.19
	3	1.53	-	-	645	329	100	51	.17
	3	2.26	-	-	229	172	50	38	.22

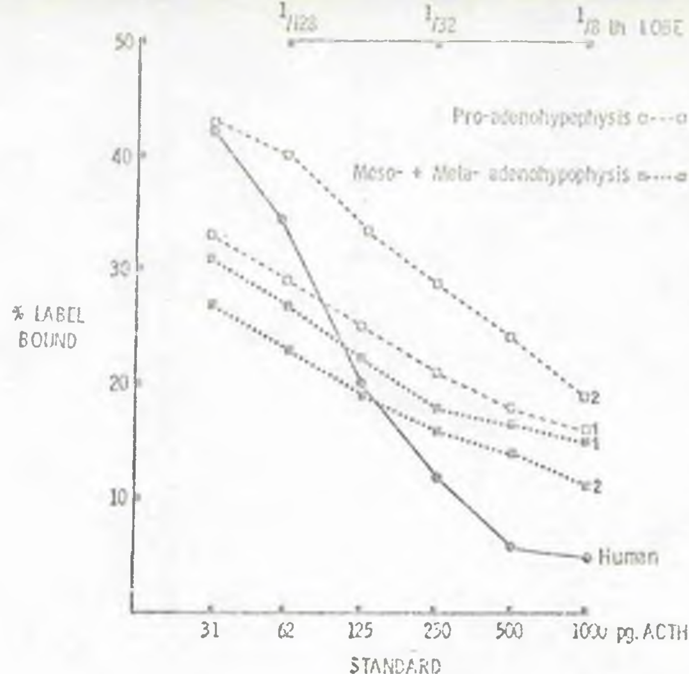


Figure 12a : Competition curves of lamprey pituitary lobe extracts using 1-24 AB. The percentage of labelled human ACTH bound to antibody is plotted on the ordinate. The amounts of reference standard are plotted on a logarithmic scale as ngs of hormone, and the amounts of tissue are also plotted on a logarithmic scale as a fraction of the total lobe.

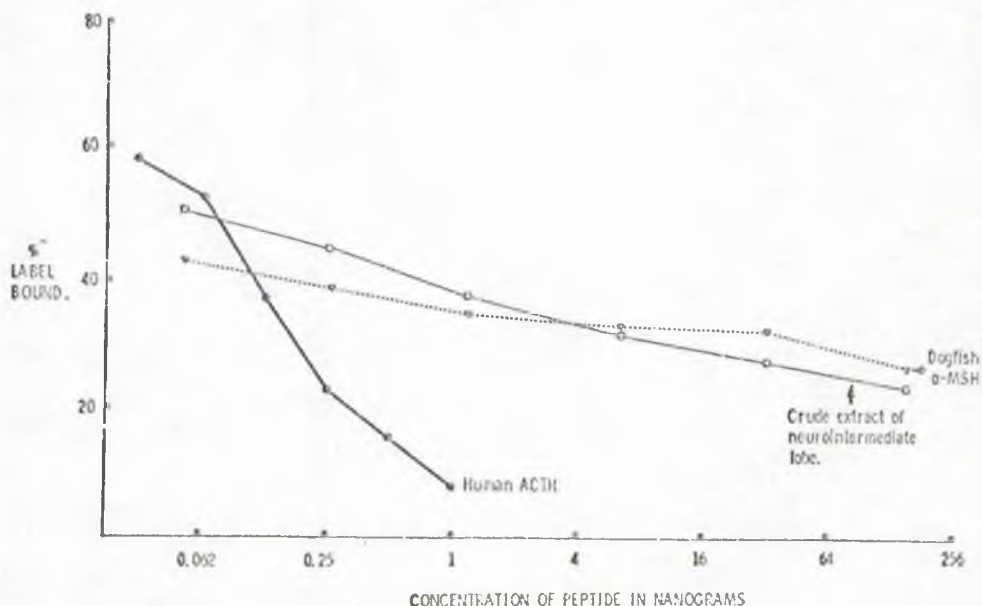


Figure 12b : Competition curves of purified dogfish α-MSH and crude dogfish neurointermediate lobe extract in the ACTH radioimmunoassay with 1-24 AB. The percentage of label bound to antibody is plotted on the ordinate. Amounts of human ACTH, dogfish ACTH and the crude extract are plotted on a logarithmic scale.

SECTION 3

STUDIES ON THE LOCALISATION OF ACTH IN THE RAT PITUITARY

a) COMPARISON OF IMMUNOACTIVE ACTH CONCENTRATIONS IN SEPARATED PITUITARY LOBES.

One of the most striking observations made during the comparative study was that pituitary extracts of several species (e.g. gecko, rat and guinea-pig) contained considerably more C- than N-terminally immunoactive ACTH. A comparison of the C/N ratios of anterior lobe extracts to that of a whole pituitary extract (Table 7) suggested that the posterior lobe was the source of the excess C-terminal activity. This was confirmed by assay of separated posterior lobes. The content of N- and C-terminally immunoactive and bioactive ACTH in anterior and posterior lobes of the rat pituitary are shown diagrammatically in Fig. 13. The mean ACTH concentration (μg/g wet weight tissue) in anterior lobes was: N= 105 (range 64-176), C = 95 (46-142), Bio = 50 (22-99). C/N ratio = 0.9 (0.7-1.2). In the posterior lobes the values were: N = 63 (30-94), C = 2192 (852-4634), Bio = 18 (14-23), C/N ratio = 32 (18-58). Posterior lobe extracts contained minimal bioactivity and N-terminally immunoactive ACTH in comparison to C-terminal activity, and thus appeared to contain a peptide which was immunologically and biologically distinct from human α^{1-39}ACTH. The anterior lobes extracts had approximately equal C and N-terminal ACTH levels, suggesting that rat ACTH is similar to α^{1-39}ACTH.

There was a marked C/N dissociation in guinea-pig posterior pituitary lobe extracts (mean $>$ 55) (Table 7) and also in pig posterior pituitary powder extracts, and pitressin (Parke-Davies) (Table 8).

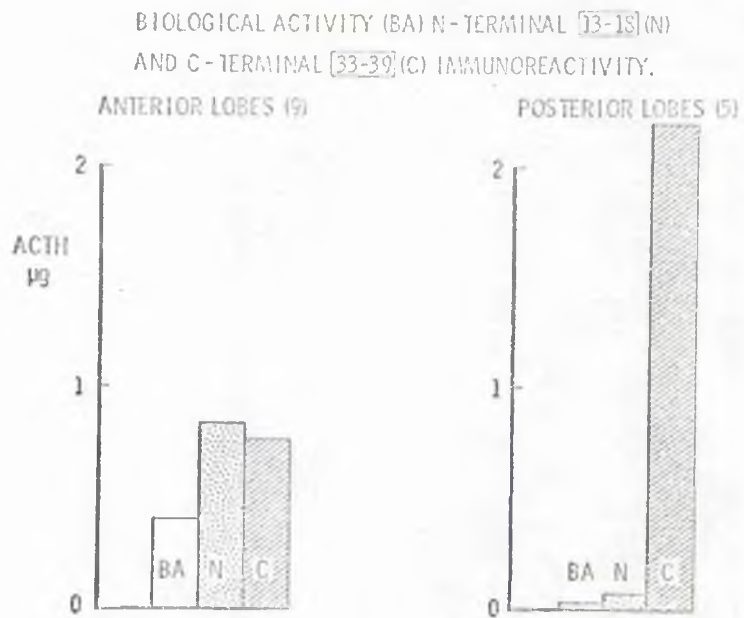


Figure 15 : Total ACTH activity in rat pituitary lobes. Content of HCl extracted lobes - biological activity(BA) , N-terminal(N) and C-terminal(C) immunoactivity.

Immunoactivity:- (ng ACTH per mg powder)

SOURCE	N-terminal	C-terminal	C/N ratio
Paines & Byrne			
RF 2390	1726	8047	4.7
RF 2620	378	3645	9.6
RG 2020	411	7009	17.0
Ferring's	1449	7954	5.5
Piton snuff (Armour's)	480	2260	4.7
Pitressin (Parke-Davies)	162 ng/ml	11578 ng/ml	71.5

Table 8 : C- and N-terminally immunoactive ACTH concentrations in pig posterior pituitary lobe powders (acetone-dried) and pitressin , demonstrating the preponderance of C-terminal activity.

b) ORGAN CULTURE OF RAT PITUITARIES

Experiments were undertaken to determine whether the C-terminally immunoactive substance found in posterior lobe extracts was released into organ culture.

(i) The separated lobes of eight freshly dissected pituitaries were placed in individual organ culture dishes. The media were removed and assayed after two days with the N- and C-terminal antisera (Fig. 14a). The mean levels of N- and C-terminally immunoactive ACTH released into the medium by anterior lobes were respectively 36 (range 24-50) and 40 (17-74) ng, with a mean C/N ratio of 1.1 (0.6-1.9). The mean levels released by the posterior lobes were respectively 14 (10-21) and 193 (138-399) ng, with a mean C/N ratio of 13.7 (10.3-19.1). These results indicate that C-terminally immunoactive ACTH can be released from posterior lobes *in vitro*.

(ii) Seven of the lobes were maintained in organ culture for 15 days. The media were changed after 2, 8 and 15 days, and on the 15th day, tissues were removed, extracted in 0.1 N hydrochloric acid and assayed. The cumulative concentrations of ACTH released by both lobes are shown diagramatically in Fig. 14b. The posterior lobes continued to release C-terminally immunoactive material at a high rate. N-terminal ACTH levels were at the limit of detection of the assay (c. 5 ng/day). Anterior lobes released approximately equal amounts of C- and N-terminally immunoactive ACTH. The mean ACTH content in the tissues after fifteen days was: anterior lobes (2) N = 281 ng, C = 195ng, C/N ratio = 0.7, and posterior lobes (3) N = 10 ng, C = 330 ng, C/N ratio = 33.0 (cf. fresh tissues).

(iii) Two pituitaries were maintained for fifteen days with Trasylol added to the medium (500 KIU/ml). There was no apparent effect on the dissociation of C or N-terminal activity in either anterior or posterior lobe cultures.

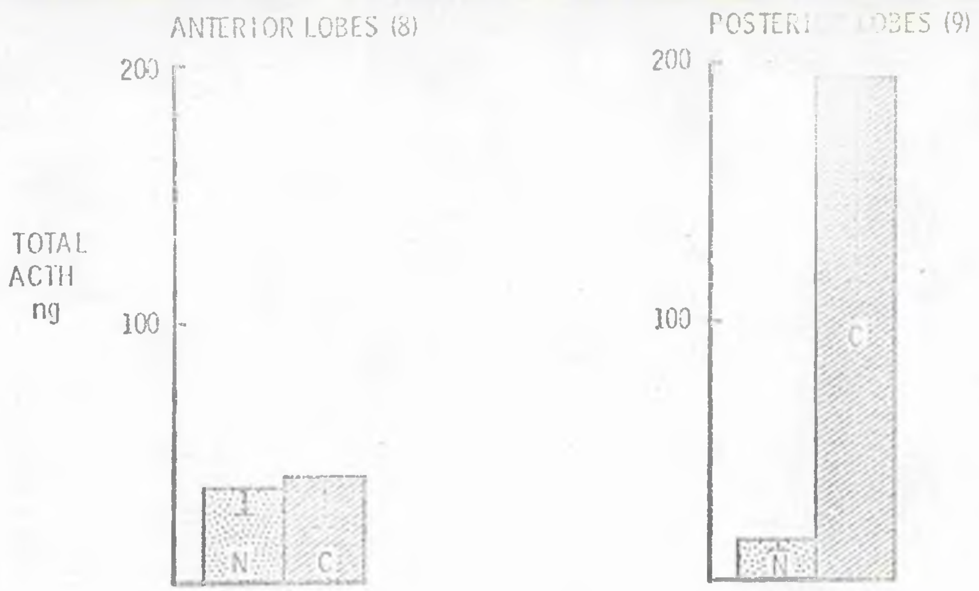


Figure 14 a : Total N- and C-terminally immunoactive ACTH released by rat pituitary lobes maintained in organ culture for [illegible] hours.

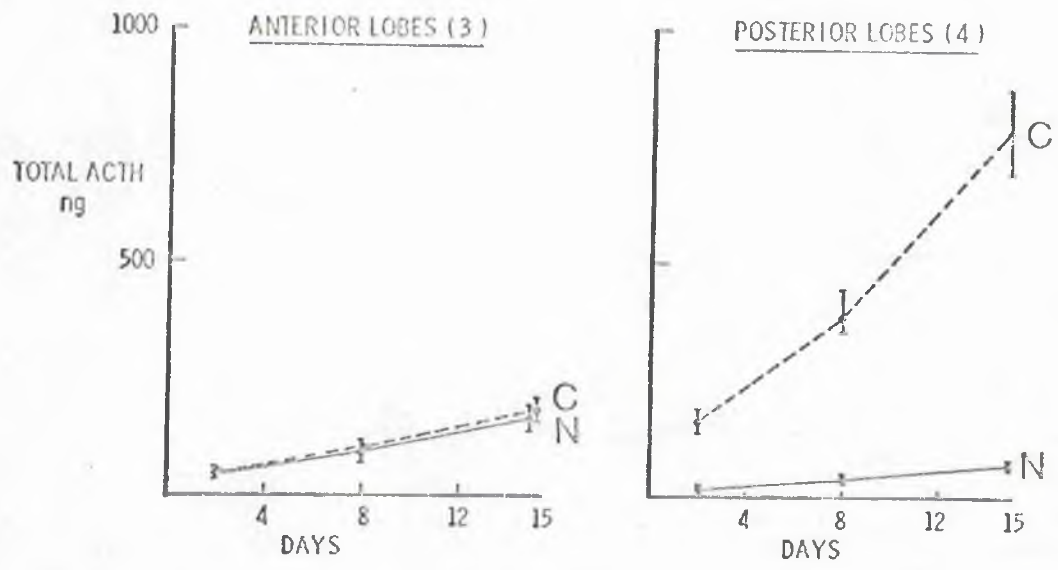


Figure 14 b : Cumulative levels of ACTH released by rat anterior and posterior pituitary lobes kept in organ culture for 15 days. Total ACTH released is shown on the ordinate in ng , and the time in days is plotted along the abscissa. Media were changed 2,8, and 15 days after the start of the cultures and assayed for N-(1-10 AA) and C-([illegible] AA) terminal activity.

c) IMMUNOFLUORESCENT STAINING OF RAT PITUITARIES

The immunofluorescent localization of C-terminally immunoactive material in the rat pituitary is shown in illustration 1a. With the 33-39 AB, at dilutions of 1/4-1/32, the pars intermedia shows up as a bright fluorescent band, and all its cells appear to stain. The pars nervosa is distinguished by a lack of significant fluorescence. The pars distalis shows patchy fluorescence, which may indicate the position of the rat corticotrophin-producing cells.

Sections stained with rabbit human growth hormone antiserum (illustration 1b), which was used as a control, showed fluorescence only in cells of the pars distalis. The pars intermedia did not stain.

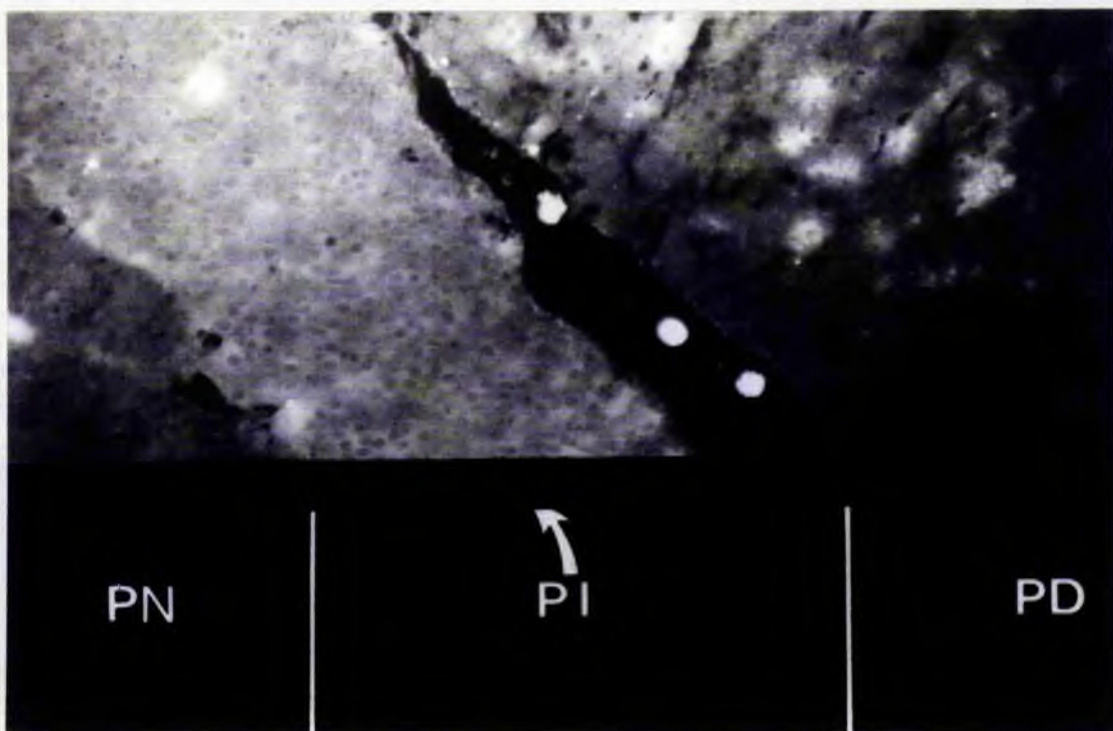


Illustration 1 : Rat pituitary section immunostained with 35-39 AB (1/4 dilution). Positive reaction with all cells of the pars intermedia (PI) and scattered cells in the pars distalis (PD). The black area is the pituitary cleft separating the two lobes of the pituitary. Pars nervosa(PN) is on the left of the picture.

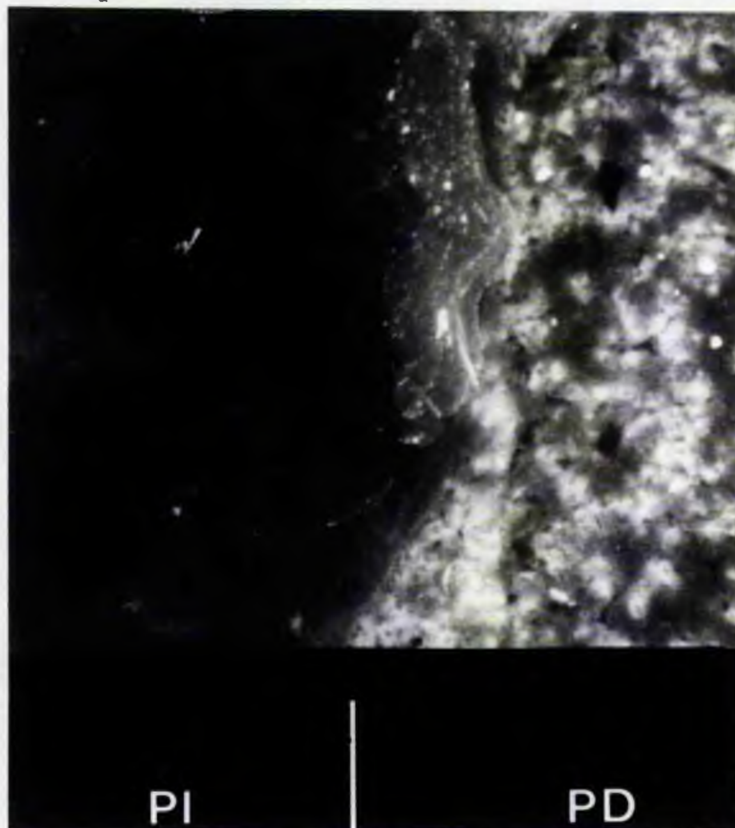


Illustration 2: Rat pituitary section immunostained with anti-human growth hormone serum (1/16 dilution). Positive reaction with many cells in the pars distalis (PD) , but no reaction with pars intermedia (PI) or pars nervosa.

SECTION 4

PURIFICATION AND CHARACTERISATION OF ACTH-LIKE PEPTIDES IN RAT, PIG AND MAN

a) RAT

(i) Pilot experiments Four rat posterior pituitary lobes were extracted in 0.1N hydrochloric acid, and the extract (containing approximately 10 µg C-and 0.25 µg N-terminally immunoactive ACTH) was submitted to gel filtration on Biogel P6 (C.S. 3). Synthetic α-MSH and ACTH (Ferring's, Malmo) were applied to the same column before and after chromatography of the extract. Fractions were collected and assayed for C-terminal activity (Fig.15) and three active peaks were found - a small peak in the void volume, a peak corresponding to the position where the ACTH eluted, and a large peak eluting between the ACTH and α-MSH regions.

In a further experiment, whole pituitaries from two hundred rats were extracted with 15% trifluoroacetic acid. The total ACTH content of the extract was: bioassay - 25 µg; N-terminal immunoassay 40 µg; C-terminal immunoassay - 350 µg. On Biogel P6, the extract gave the elution pattern shown in Fig. 16. The major peak of C-terminal activity was found in fractions 22 to 30, and it was shown by assay that this region did not contain MSH or ACTH bioactivity. Fractions 25 to 28 were applied to CM-cellulose and 80% of the activity washed out immediately. This was reapplied to DEAE-cellulose, and step elution yielded a single peak of active material. Its amino acid composition was:

ASX	SER	GLX	PRO	GLY	ALA	VAL	LEU	TYR	PHE	LYS	ARG
2.8	1.3	6.1	2.8	2.1	3.2	2.4	1.0	0.8	1.1	0.9	1.1

This analysis showed a striking resemblance to the 18 to 39

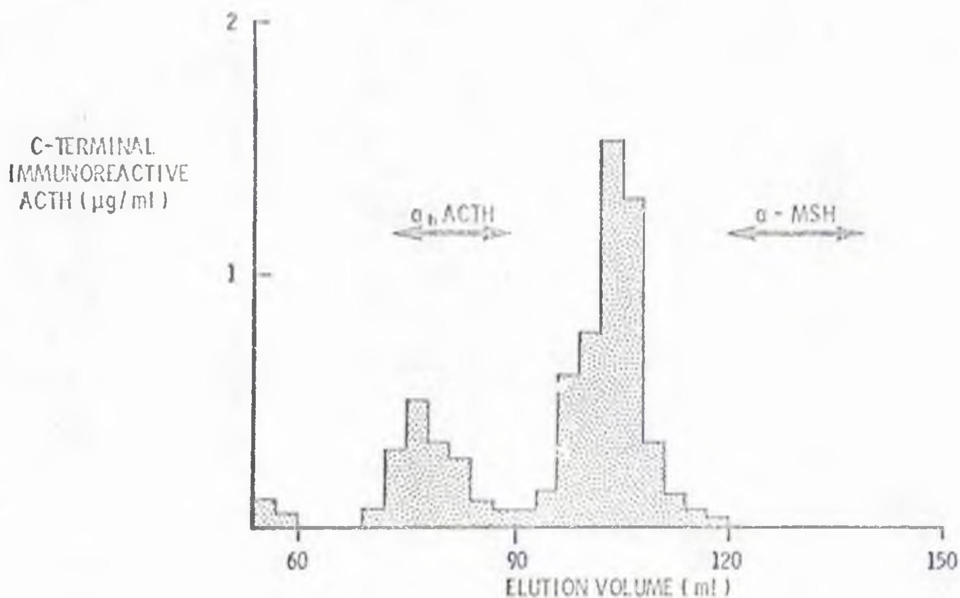


Figure 15 : Gel filtration of a 0.1 N hydrochloric acid extract of four rat posterior pituitary lobes on Biogel P6 (C.S.5). The column was developed at 20° with 5% acetic acid at a flow rate of 9 ml per hour. Three ml fractions were collected and assayed for C-terminal activity. 3 mg each of Ferring's synthetic human ACTH and α-MSH were chromatographed before and after gel filtration of the extract and were detected in the eluate by measurement of their UV absorbtion at 280 nm.

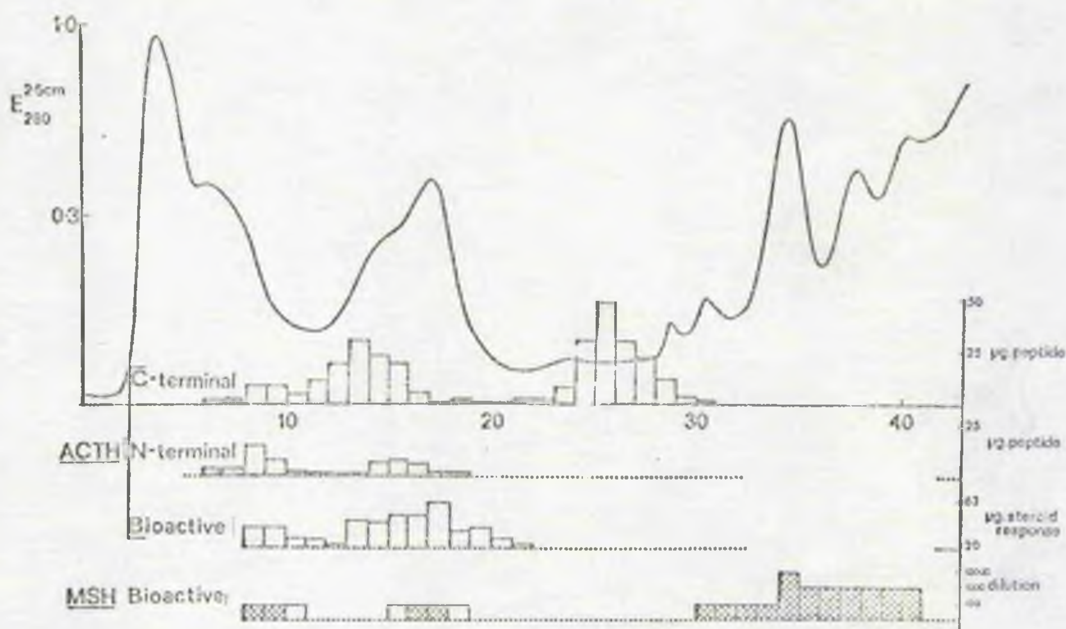


Figure 16 : Gel filtration of the 15 °/₀ trifluoroacetic acid extract of 200 rat pituitaries on Biogel P6. The column (1 cm x 100 cm) was developed at 20° with 5°/₀ acetic acid at a flow rate of 6 ml per hour. Twenty minute fractions were collected and monitored continuously at 280 nm (solid line). ACTH C- and N-terminal immunoactivity are shown by the two upper rows of shaded blocks (µg ACTH per fraction) and ACTH bioactivity is shown by the third row of shaded blocks (µg corticosterone *in vitro* response by isolated rat adrenal cells incubated with 1/40,000 dilution of each fraction). MSH activity is shown by the bottom row of blocks (representing the greatest dilutions at which fractions will still effect darkening of frog skin) .

portion of ACTH, although several amino acids were present in excess.

(ii) Purification of C-terminally immunoactive material and rat MSH

Whole pituitaries from 140 rats were dried in acetone (320 mg powder), homogenised twice with glacial acetic acid, the extracts pooled and freeze-dried, and the resulting powder (150 mg) reconstituted in 5% acetic acid and submitted to gel filtration on Biogel P6 (C.S.3)(Fig.17). 95% of the N-terminal immunoactivity was found in fractions 45 to 50, and this corresponded to a peak of C-terminal activity (ACTH region). The major C-terminally immunoactive peak (300 μg) was found in fractions 54 to 64. MSH bio-activity was found only in fractions 67 to 78.

1) C-terminally immunoactive material:

Fractions 57 to 62 were freeze-dried, reconstituted in 10 mM Me_3NAc (pH 5) and applied to DEAE-cellulose (C.S.4), which was developed with a gradient of 10 mM to 200 mM Me_3NAc (pH 5) (Fig. 18a). Two peaks of C-terminally immunoactive material were resolved, and one third of each was analysed for its amino acid composition. The first peak was heavily contaminated, but the amino acid composition of the second peak as determined is shown in Table 9. This resembled human α^{18-39}ACTH, except in the absence of a glycine and the presence of an extra valine residue. Approximately 37 μg of the peptide was isolated.

2) MSH-like material:

Fractions 74 to 76 of the Biogel P6 run were applied to CM-cellulose (C.S. 5) in 10 mM Me_3NAc (pH 5), and eluted with a gradient of 10 to 600 mM Me_3NAc (pH 5) (Fig. 18b). The three active peaks were analysed for their

amino acid composition. The major peak (total [illegible] µg) gave the following analysis, which resembles that of α-MSH isolated from other mammals:

SER	GLX	PRO	GLY	VAL	MET	TYR	PHE	LYS	HIS	TRY	ARG
1.7	1.1	1.0	1.1	1.1	1.0	1.0	0.9	1.2	1.2	(1)	1.3

The first peak possibly contained contaminating α-MSH, as analysis revealed the following amino acid ratios, which are characteristic of oxytocin (confirmed by a specific radioimmunoassay for oxytocin):

ASX	GLX	PRO	GLY	½/CYS	ILE	LEU	TYR
1.0	1.2	1.3	1.1	1.4	1.0	1.0	1.0

No amino acids were detected in fractions 27/28, which suggests that these tubes may have been contaminated during assay.

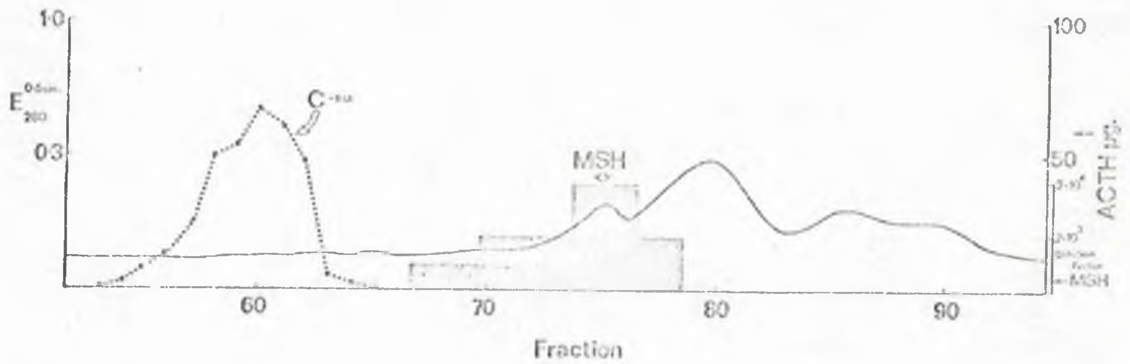


Figure 17 : Gel filtration of the glacial acetic acid extract of 140 rat pituitaries on Biogel P6 (C.S.3). The column was developed at 20° with 5°/₀ acetic acid at a flow rate of 6 ml per hour. After eluting 54 mls (fraction 27) twenty minute fractions were collected and monitored at 280 nm (solid line). ACTH N-terminal activity and part of the C-terminal activity were found in fractions 45 to 50. The second half only of the trace is included in the figure. C-terminal ACTH activity is shown by the dotted line and MSH activity by the shaded areas.

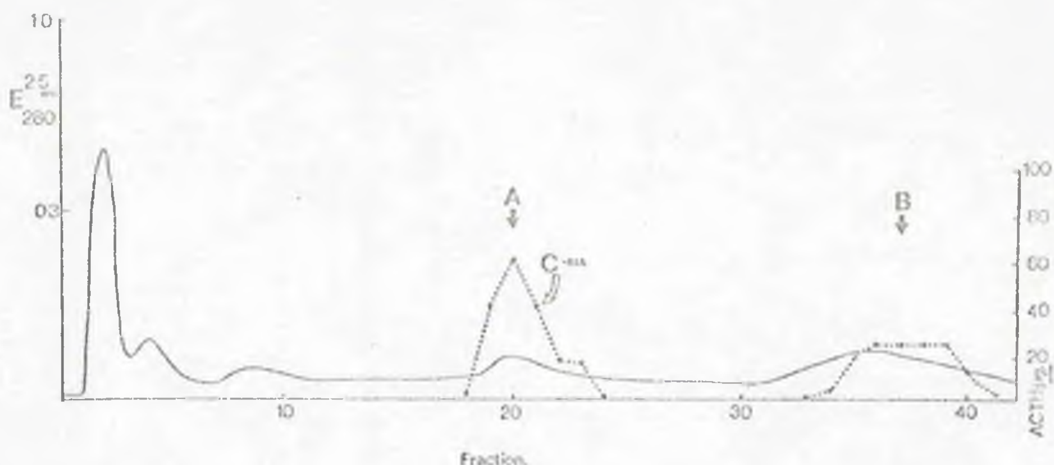


Figure 18 a : Ion-exchange chromatography of C-terminally immuno-active material from the rat pituitary (after purification on Biogel P6) on DEAE-cellulose. The column (0.6 cm x 15 cm) was developed with a gradient of 10mM to 200 mM trimethylamine acetate (pH5) at a flow rate of 1.5 ml per hour. Twenty minute fractions were collected and monitored for absorbance at 280 nm (solid line). ACTH C-terminal activity is shown by the dotted line.

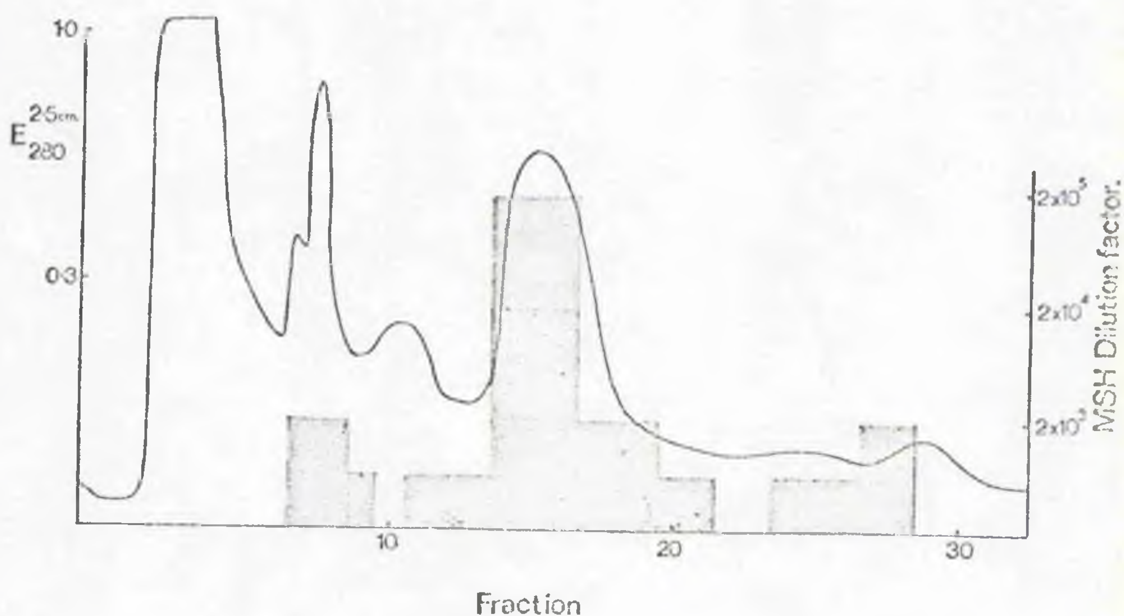


Figure 18 b : Ion-exchange chromatography of MSH active material from rat pituitary on CM-cellulose. The column (0.6 cm x 10 cm) was developed with a gradient of 10mM to 600 mM trimethylamine acetate (pH5) at a flow rate of 1.5 ml per hour. Twenty minute fractions were collected and monitored for absorbance at 280 nm (solid line). MSH activity is shown by the shaded areas.

b) PIG

(i) <u>Preliminary experiments to compare several extraction methods for the purification of porcine C-terminally immunoactive peptides</u>

Several of the porcine posterior pituitary lobe extracts were reconstituted in 5% acetic acid and chromatographed on Biogel P6.

1) Glacial acetic acid:

The powder (180 mg: C- and N-terminal activity- 2.8 mg and 0.6 mg respectively) was chromatographed on Biogel P6 and gave the elution pattern shown in Fig. 19a. The C-terminal activity was recovered in two peaks, containing 300 µg and 800 µg activity respectively. N-terminal activity was recovered in the same fractions as the first peak of C-terminal activity. The second and third peaks of MSH bioactivity probably correspond to β-MSH and α-MSH, and are included to indicate the approximate molecular size of the immunoactive ACTH components. The C-terminally immunoactive material in fractions 19 to 21 was further purified on DEAE-cellulose with a gradient, and the elution pattern was similar to that in Fig. 22. The amino acid composition of the major active peak is shown in Table 9 (peptide P3).

2) HCl/acetone:

Gel filtration of the HCl/acetone extract (61.1 mg: C- and N-terminal immunoactivity - 0.64 mg and 0.24 mg respectively) gave the elution pattern shown in Fig. 19b. Two peaks of C-terminal activity were resolved, the first of which corresponded to the N-terminal immunoactivity. They assayed as 220 µg and 300 µg ACTH respectively. No further purification was attempted.

3) 5% acetic acid:

Gel filtration of the 5% acetic acid extract (241 mg: C- and N-terminal immunoactivity - 0.5 mg and 0.4 mg respectively) gave the elution pattern shown in Fig. 19c. There was a peak of N-terminal activity in the expected position, but only one small peak of C-terminal activity was obtained corresponding to the N-terminal peak. A second very small peak of C-terminally immunoactive material was present in the low molecular weight range.

4) TFA/acetone:

The TFA/acetone extract (120 mg: C- and N-terminal immunoactivity - 2 mg and 0.5 mg respectively) gave an elution pattern and distribution of activity similar to that given by the glacial acetic acid extract (Fig. 19a). 250 μg N-terminal activity and 350 μg C-terminal activity were recovered in the first peak and 1300 μg C-terminal activity was recovered in the second peak. MSH activity was not assayed.

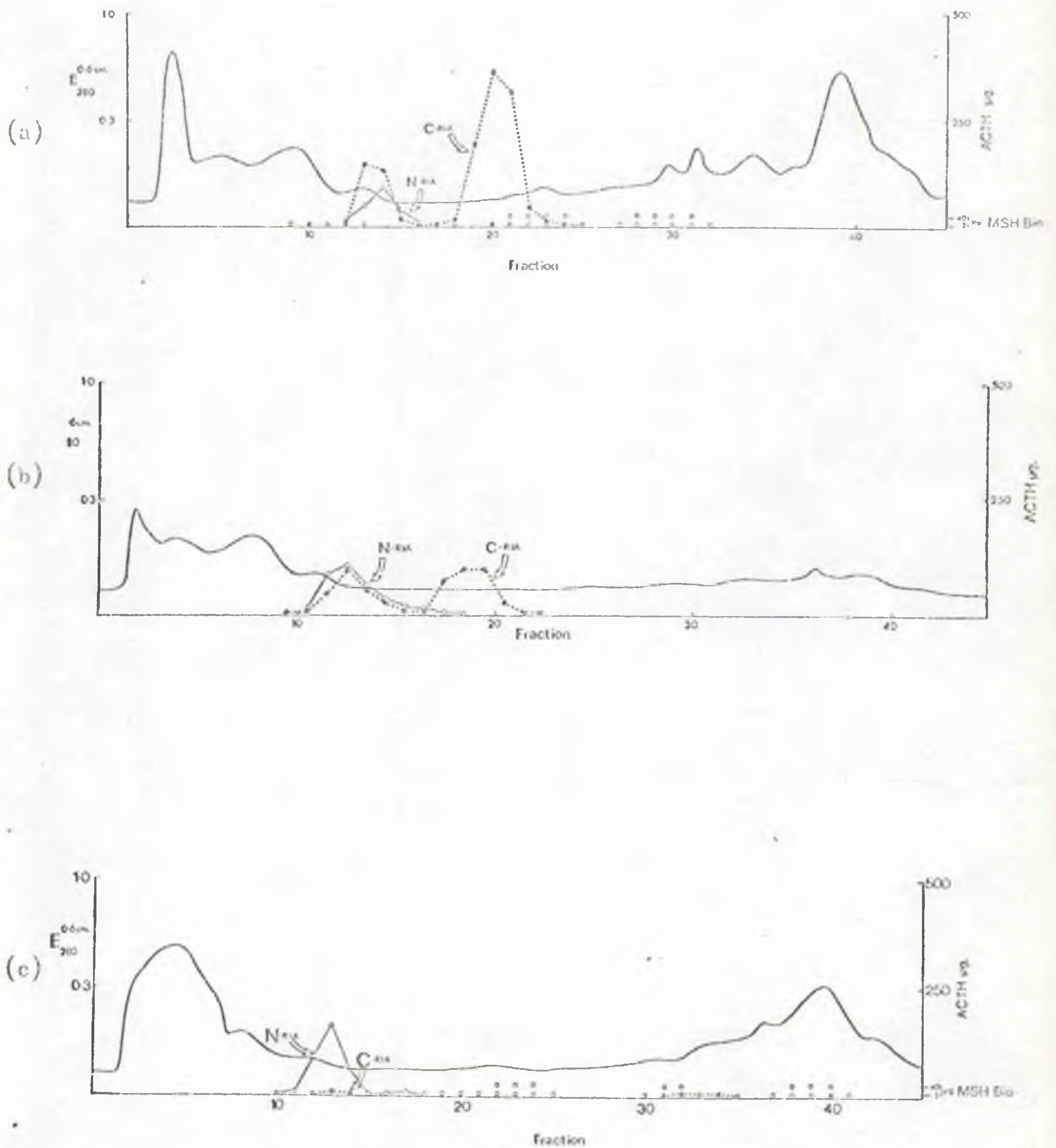
5) Other extractions:

Extraction of porcine posterior lobe powder by the method of Rosenberg and his colleagues (1961), and of fresh pig pituitaries in 15% trifluoroacetic acid, followed by gel filtration and ion-exchange chromatography, yielded small quantities of the C-terminally immunoactive material (Table 9 - peptides P4 and P5). However, these methods were unsuitable because of low final yields of the peptide.

(ii) Large scale purification of immunoreactive peptides from porcine posterior pituitary lobe powder

Ten g of posterior lobe powder was extracted

(a)



(b)

(c)

Figure 19 : Gel filtration of porcine posterior lobe extracts on Biogel P6 (C.S.5). The column (1.5 cms x 30 cms) was developed with 5% acetic acid at a flow rate of 9 mls per hour. Twenty minute fractions were collected and monitored at 280nm (solid line). C-terminal activity (dotted line), N-terminal activity (unbroken line) and MSH bioactivity (shown by squares indicating those fractions containing activity equivalent to 2 or 40 µg α-MSH) were determined. a) Glacial acetic acid extract, b) HCl/acetone extract, c) 5% acetic acid extract.

with TFA/acetone, which yielded 1.43 g dried pow[illegible] containing 23 mg C-terminally immunoactive ACTH. This was reconstituted in 15 ml 5% acetic acid and submitted to gel filtration on Biogel P2 and P6 (C.S.1). The elution pattern and distribution of C-terminal activity are shown in Fig.20. The fractions containing active material were freeze-dried, reconstituted in 4 ml acetic acid and re-chromatographed in two batches on Biogel P6 (C.S.3) (Fig.21). This second filtration step resolved the C-terminally immunoactive ACTH into two peaks. The second major peak from each of the two runs was freeze-dried, reconstituted in 10 mM Me Me_3NAc (pH 5) and applied to DEAE-cellulose (C.S.4), which was developed with a gradient of 10 mM to 200 mM Me_3NAc (pH 5) (Fig.22). The bulk of activity corresponded with the major absorbance peak beyond the void. The amino acid composition of this peak is shown in Table 9 (peptides P1 and P2) and is identical to the 18-39 portion of porcine ACTH. Analysis of peak A suggested that it represented the 18-36 portion of ACTH, but there was some contamination with acidic residues. Peak C resembled peak B in amino acid composition and possibly represented deamidated 18-39 ACTH. These minor peaks were not further studied. The total yield of peak B material was 1.1 mg by weight (calculated from amino acid analysis).

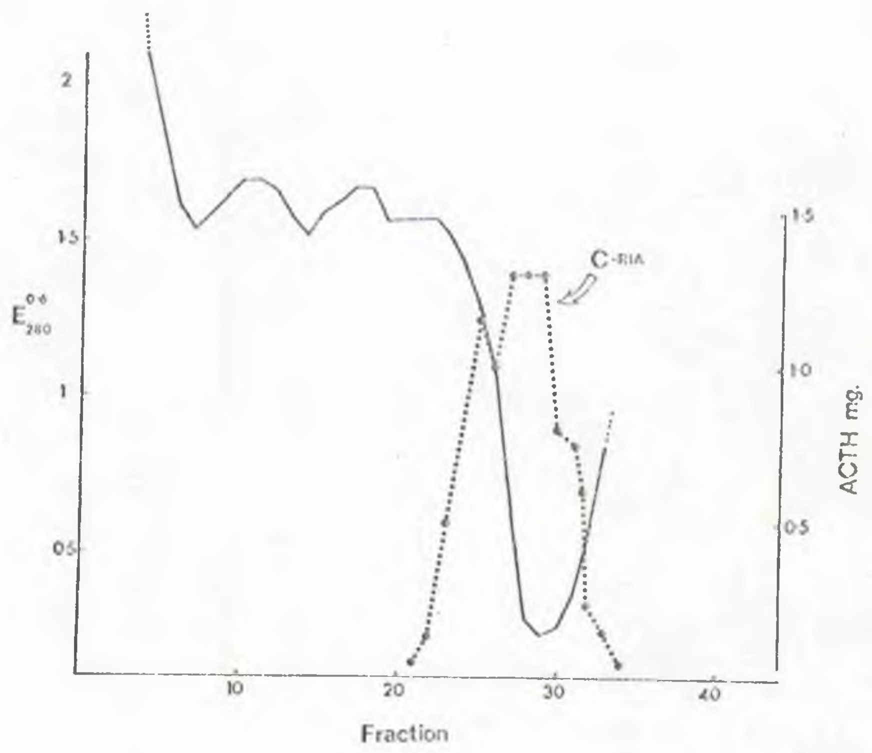


Figure 20 : Gel filtration of the TFA/acetone extract of 10 g pig posterior pituitary lobe powder on Biogel P2 and P6 (C.S.1). The columns were developed at 20° with 5°/o acetic acid at a flow rate of 15 ml per hour. Twenty minute fractions were collected and monitored at 280 nm (solid line). C-terminally immunoactive ACTH content of the fractions is shown by the dotted lines.

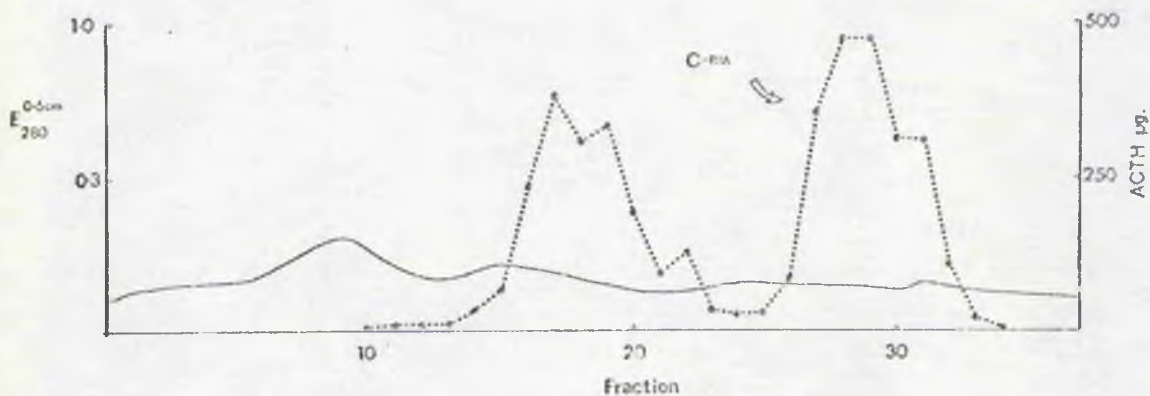


Figure 21 : Gel filtration of half of fractions 22 - 31 of the previous run (Figure 20) on Biogel P6. The column (C.3.5) was developed at 20° with 5°/o acetic acid at a flow rate of 6 mls per hour. After elution of 38 mls , 20 minute fractions were collected and monitored at 280 nm (solid line). C-terminally immunoactive ACTH is shown by the dotted line.

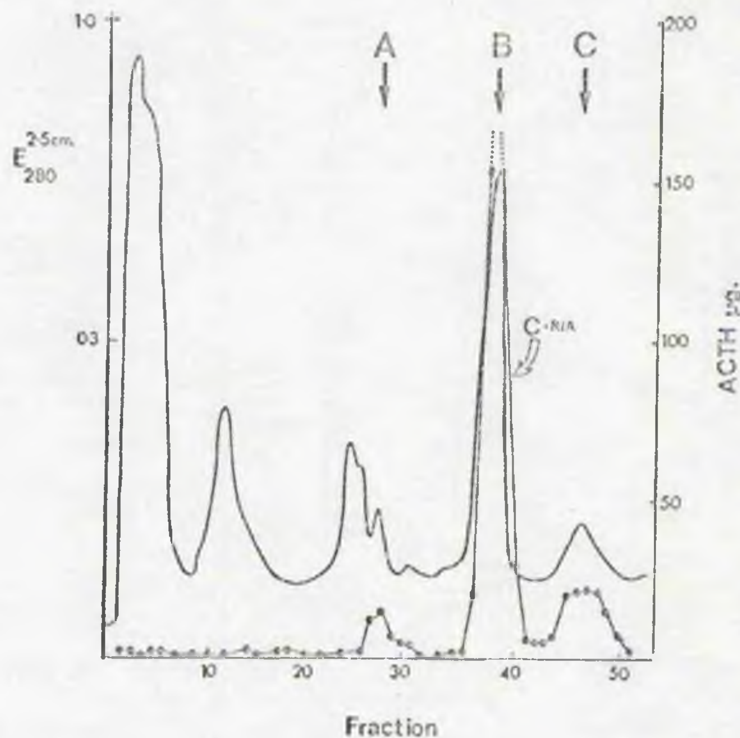


Figure 22: Ion-exchange chromatography of C-terminally immunoactive material (fractions 26 - 32) from Biogel P6 purification of porcine TFA/acetone extracted powder , on DEAE-cellulose (C.5.4). The column was developed at 20° with a gradient of 10mM to 200 mM trimethylamine acetate (both pH5) at a flow rate of 1.5 ml per hour. Twenty minute fractions were collected and monitored for absorbance at 280 nm (solid line - uninterrupted). C-terminally immunoactive ACTH is shown by the circles.

c) HUMAN TUMOUR

A bronchial carcinoid tumour, removed at operation from a 34 year old woman with the ectopic ACTH syndrome, was extracted with HCl/acetone as described. The powder was assayed for C-and N-terminal ACTH immunoactivity and bioactivity; bioassay = 0.78 µg/mg powder, N = 0.96 µg/mg, C = 53.5 µg/mg.

Ten mg of powder were reconstituted in 5% acetic acid and submitted to gel filtration on Biogel P6 (C.S.2). The elution pattern and distribution of C-terminally immunoactive and bioactive ACTH and bioactive MSH are shown in Fig.23. Almost all of the C-terminally immunoactive material was found exclusively in a single peak corresponding in position to the C-terminally immunoactive material isolated from pig and rat pituitaries. MSH activity was found in fractions corresponding roughly to the position of α-MSH and also in several fractions eluting before ACTH. This may represent larger molecular weight materials possessing MSH activity (e.g. LPH). Ion-exchange chromatography of fractions 43 to 47 on DEAE-cellulose gave an elution pattern shown in Fig.24. A portion of the active peak was taken for amino acid analysis (Table 9).

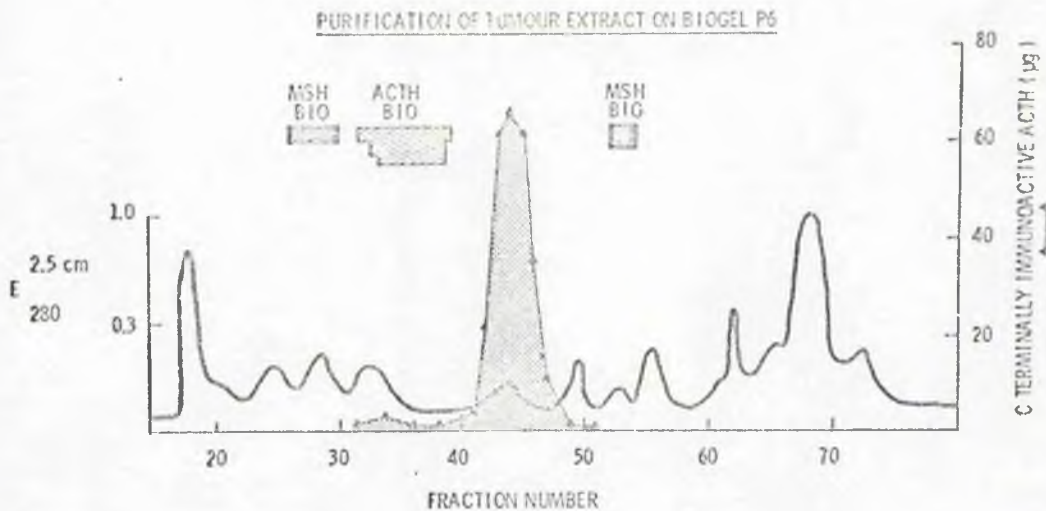


Figure 25: Gel filtration of the HCl/acetone extract of the human bronchial carcinoid tumour on Biogel P6. The column (C.S.2) was developed with 5°/o acetic acid at a flow rate of 6 mls per hour. Twenty minute fractions were collected and monitored at 280 nm (solid line). C-terminal activity is shown by the shaded area, and bioactive ACTH and MSH are shown by the blocks.

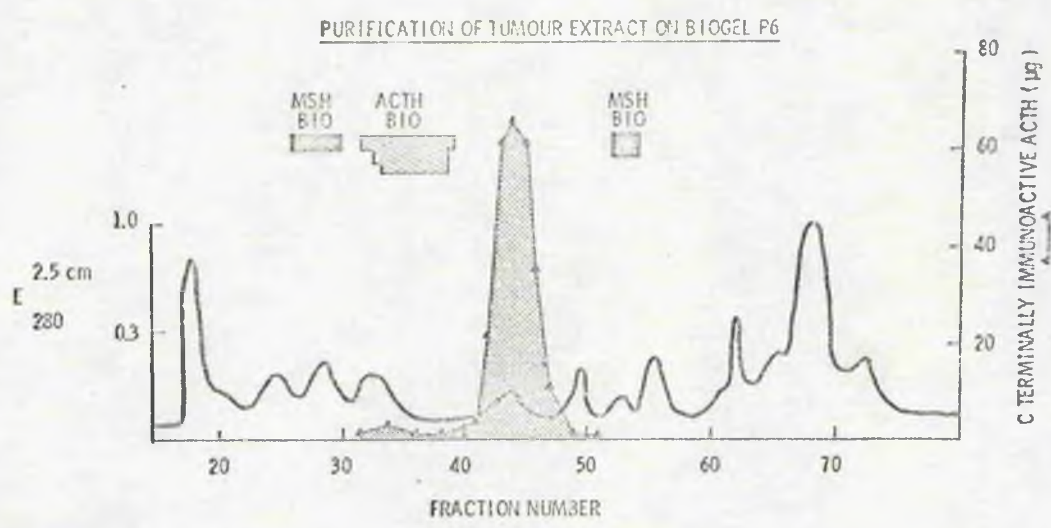


Figure 25: Gel filtration of the HCl/acetone extract of the human bronchial carcinoid tumour on Biogel P6. The column (C.S.2) was developed with 5°/o acetic acid at a flow rate of 6 mls per hour. Twenty minute fractions were collected and monitored at 280 nm (solid line). C-terminal activity is shown by the shaded area, and bioactive ACTH and MSH are shown by the blocks.

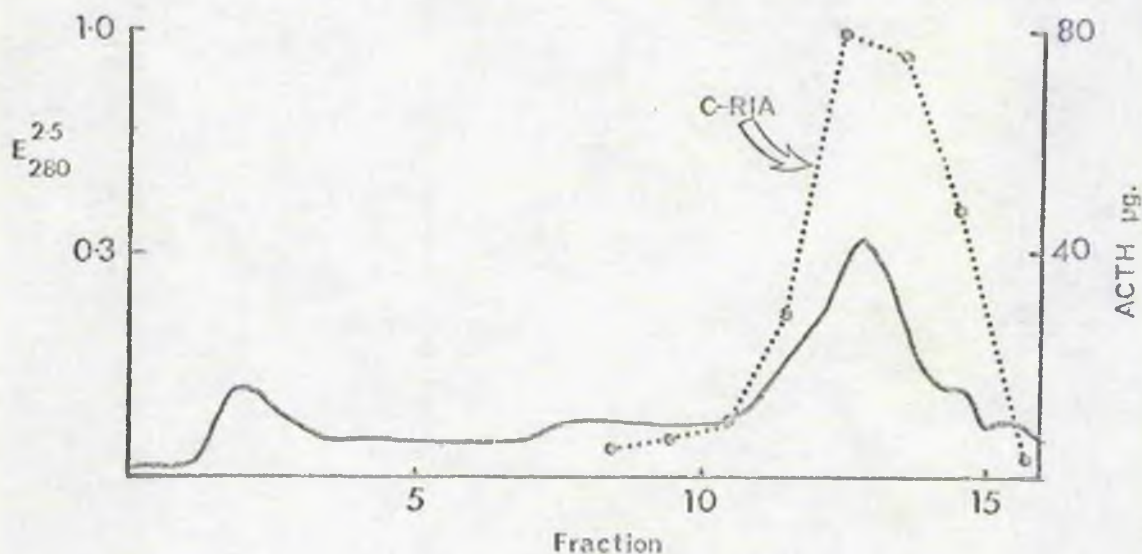


Figure 24 : Ion-exchange chromatography on DEAE-cellulose of Biogel P6 purified C-terminally immunoactive material from the human tumour. The column (C.S.4) was developed with a gradient of 10 to 600 mM trimethylamine acetate (both pH5) at a flow rate of 1.5 mls per hour. Twenty minute fractions were collected and monitored for absorbance at 280 nm. C-terminal activity is shown by the dotted line.

d) PARTIAL SEQUENCING STUDIES

(i) Investigation of NH_2-terminal sequence of porcine and tumour peptides

The purified porcine and tumour peptides were further characterised with respect to their N-terminal sequences by Edman degradation. Two steps, carried out on 20 µg of the porcine peptide removed first an arginine and then a proline residue, and one step carried out on 7 µg of the tumour peptide removed an arginine residue. The sequence Arg-Pro is present at position 18-19 of porcine and human ACTH, and the N-terminal sequencing results therefore help confirm the similarity of the two peptides to the 18-39 portion of ACTH.

(ii) Investigation of the C-terminal sequence of porcine and tumour peptides

Carboxypeptidase C digestion was carried out on 30 µg of the porcine peptide. After four hours, 0.50 nmol phenylalanine, and after 16 hours, 2.74 nmol phenylalanine, 1.10 nmol glutamic acid, 0.59 nmol leucine and 0.53 nmol proline were detected. These results are consistent with a C-terminal sequence -Pro-Leu-Glu-Phe (i.e. 36-39 ACTH). Carboxypeptidase digestion was carried out on 2.4 µg of the tumour peptide (1 nmol). After sixteen hours digestion, the following amino acids had been released: phenylalanine, 0.61 nmol; glutamic acid, 0.38 nmol; leucine, 0.32 nmol.

e) COMPARISON OF C-TERMINAL PEPTIDES WITH KNOWN CORTICOTROPHINS

The amino acid compositions of the C-terminally immunoactive peptides derived from rat, pig and human tumour are compared with the known amino acid compositions of human and porcine α^{18-39}ACTH in Table 9. Taking into account the terminal sequencing data, and the immunological identity of the peptides with the C-terminal portion of ACTH, it would be reasonable to suggest that the peptides from all three sources represent the α^{18-39} fragment of the corresponding ACTH molecule. The single amino acid difference between pig and human ACTH (a leucine-serine change at position 31) is mirrored in the composition of the pig and human peptides. The rat peptide differs from human α^{18-39} ACTH only in the absence of a glycine residue and the presence of an extra valine (the glutamic acid ratio is high, due to contamination).

AMINO ACIDS	(1)	(2)	PIG CLIPS (3)	(4)	(5)	PIG 18–39 ACTH	RAT CLIP	HUMAN TUMOUR CLIP	HUMAN 18–39 ACTH
ASX	1.7	2.0	2.2	2.1	2.3	2	2.1	2.3	2
SER	–	0.1	0.1	0.3	0.1	–	0.9	1.4	1
GLX	3.9	4.1	4.6	4.0	3.8	4	4.6	4.3	4
PRO	2.9	2.9	2.8	2.6	3.0	3	2.9	3.2	3
GLY	1.2	1.3	1.2	1.6	1.2	1	0.3	1.7	1
ALA	2.7	2.9	3.1	3.0	3.2	3	2.9	2.8	3
VAL	1.9	2.0	2.0	1.9	1.9	2	3.0	1.3	2
LEU	1.9	1.8	1.9	1.7	1.9	2	1.0	0.9	1
TYR	1.0	1.0	1.0	1.0	1.0	1	1.1	1.0	1
PHE	1.7	1.8	1.8	1.8	1.8	2	1.6	1.5	2
LYS	1.0	1.0	1.0	1.2	1.3	1	1.0	0.8	1
ARG	1.0	0.9	1.0	1.1	0.9	1	1.0	0.8	1

Table 9 : Amino acid composition of C–terminally immunoactive peptides extracted from pig and rat pituitaries and a human bronchial carcinoid tumour ; a comparison with porcine and human 18–39 ACTH.
Pig peptides:– (1) and (2) – TFA/acetone extracted preparations, (3) – glacial acetic acid extracted preparation, (4) – 0.1N hydrochloric acid extracted preparation, (5) – 15°/o TFA extracted preparation.

DISCUSSION

Comparative studies

The availability of a bioassay and of radioimmunoassays, with antisera specific for different parts of the ACTH molecule, enabled a survey of corticotrophin in several vertebrates using crude pituitary extracts. Although the precise sequence of corticotrophins could not be established by this approach, it had the advantage of allowing a rapid comparative assessment in a large variety of species. All extracts tested contained ACTH bioactivity, and material which cross-reacted with the 13-18 AB. The slopes of the displacement curves with the 13-18 AB paralleled those of the human standard over some portion of the curve in all species except dogfish and lampreys. This suggests that the biologically important region of the ACTH molecule, constituting the sequence of basic amino acids in positions 15 to 18 of mammalian ACTH, is similar in the range of vertebrates from teleosts to mammals, but is slightly altered in the more primitive elasmobranchs, and possibly absent in the lamprey (see below). Partial or absent cross-reactions of pituitary extracts with the 14-24 AB and 17-39 AB imply differences between the more C-terminal portions of vertebrate corticotrophins. This is supported by the studies using the C-terminally directed antiserum 33-39 AB, with which all fish and amphibian extracts failed to cross-react, and all reptilean, avian and mammalian extracts gave displacement curves indistinguishable from those given by human ACTH - indicating that all the higher vertebrates studied possess the peptide sequence from 33 to 39 of mammalian ACTH. The absence of cross-reactivity in the fishes and amphibians suggests

either that their ACTH lacks this sequence (i.e. a shorter molecule) or has a considerably altered sequence (i.e. immunological identity is destroyed).

Despite the presence of ACTH bioactivity and N-terminal immunoactivity in lamprey extracts, the levels (expressed as ng ACTH per g body weight) are exceedingly low compared with other vertebrates and it is doubtful whether they can be considered to be a significant demonstration of ACTH in lamprey pituitaries. The absence of ACTH would not be unreasonable in view of the lack of convincing evidence for a pituitary-adrenal axis in these animals (Weisbart & Idler, 1970; Hardisty, 1972). The fact that significant amounts of immunoactive material can be demonstrated with the 1-24 AB can probably be ascribed to an α-MSH-like substance, in view of the cross-reaction of this antiserum with mammalian and dogfish α-MSH and the preponderance of this material in the posterior half of the lamprey pituitary.

Dissociation of C- and N-terminally immunoactive ACTH

An excess of C- to N-terminal immunoactivity was found in rat, guinea-pig and pig posterior pituitary lobe extracts and whole pituitary extracts of geckos. It can be concluded on the basis of the studies reported in this thesis that the bulk of this excess C-terminal activity in the rat and pig posterior lobes is due to a pituitary peptide resembling the α^{18-39} portion of ACTH in physicochemical and immunological characteristics and amino acid composition. Studies carried out on the rat pituitaries indicated that this peptide was localised in the cells of the pars intermedia and could be released *in vitro*.

The purified peptides

The peptide isolated from the rat pituitaries contained 22 amino acid residues and had an amino acid composition almost identical to that of the α^{18-39} portion of human ACTH. It differed from human α^{18-39} in possessing an extra valine, and lacking a glycine residue. This suggests a glycine-valine replacement at position 26 of ACTH. This replacement is possible with only one DNA base change (e.g. GGU-GUU). It is of interest that the amino acid composition of a mouse pituitary tumour ACTH, published by Canfield and his colleagues (1970), has an extra valine and lacks a glycine in comparison with porcine ACTH.

The pig peptide was identical in amino acid composition to porcine α^{18-39}ACTH. Sequencing studies showed that the N-terminal amino acids were Arg-Pro-(i.e. α^{18-19}ACTH) and the C-terminal ones were -Pro-Leu-Glu-Phe (i.e. α^{36-39}ACTH). More recently, it has been demonstrated by analysis of tryptic and peptic digests that the sequence of amino acids in the pig peptide is identical to that of porcine α^{18-39}ACTH.

The tumour peptide

The demonstration that certain tumours associated with the ectopic ACTH syndrome contain more C- than N-terminal immunoactivity (Orth et al., 1968; Ratcliffe et al., 1972) has remained a puzzling observation for several years. This thesis provides an indication, however, that the bulk of this excess C-terminal activity can be accounted for, in the majority of tumours, by a peptide resembling the C-terminal peptides isolated from the rat and pig as regards molecular weight (Orth et al., 1971), amino acid composition and terminal amino acids.

Proposed terminology for the C-terminally immunoactive peptide

It is concluded, on the basis of these findings, that the excess C-terminal activity found in the pars intermedia of the rat and pig pituitaries and in certain human tumours is due to the presence of a peptide resembling the α^{18-39} portion of ACTH of the appropriate species. It is proposed that this peptide be designated 'Corticotrophin-Like Intermediate Lobe Peptide' - (CLIP).

Mechanism of formation of CLIP

It is proposed that CLIP and α-MSH are derived from ACTH produced intracellularly and cleaved to produce two fragments, one of which, on acetylation and amidation, becomes α-MSH and the other CLIP (Fig.25). All three peptides are thus products of a single gene. The site of ACTH cleavage is not known, but may be between residues 16 and 17 (Lys-Arg) or 15 and 16 (Lys-Lys) with subsequent carboxypeptidase action to residue 13, or between residues 13 and 14 (Val-Gly) by hydrolytic transamidation (Geschwind, 1967). Sequential amino-peptidase action on the C-terminal fragment would be prevented by the Arg-Pro sequence at positions 18 and 19. Evidence for this hypothesis is summarised in the following paragraphs.

First, both α-MSH and CLIP should be demonstrable in the same cell. Specific immunostaining methods have shown that α-MSH is localised in the pars intermedia of the rat (Hess *et al.*, 1968) pig, cow and sheep (Dubois, 1972). CLIP has also been localised by these methods in the rat and CLIP and α-MSH probably originate in the same cell because staining of the pars intermedia cells is essentially uniform.

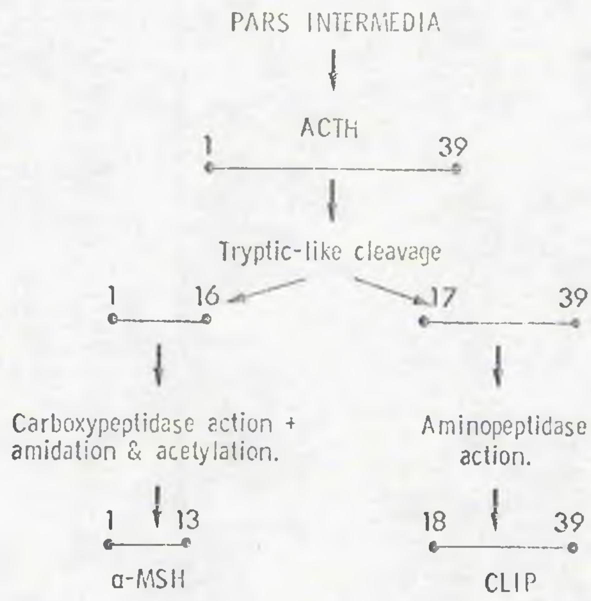


Figure 25: Proposed mechanism for the formation of α-MSH and CLIP from ACTH in the pars intermedia cell.

Second, α-MSH and CLIP should be identical in amino acid sequence to the appropriate portions of the homologous ACTH. Rat α-MSH is similar in amino acid composition to the known mammalian α-MSHs, which are identical to the N-terminal tridecapeptide sequence of the corresponding corticotrophins. Pig CLIP is identical in amino acid composition terminal sequences, and tryptic and peptic fragments to pig α^{18-39}ACTH, and rat CLIP resembles the α^{18-39}portion of human ACTH and a mouse tumour ACTH.

Third, the amounts of α-MSH and CLIP should be approximately equal on a molar basis. In rat pituitaries, approximately equivalent quantities of CLIP and α-MSH were isolated. The yields of CLIP obtained in the pig are high, as are those for α-MSH reported by other workers. Furthermore, in man, post-mortem pituitaries contain low levels of CLIP (unpublished observations) similar to the low levels reported for α-MSH (Abe _et al._, 1967).

Fourth, the pars intermedia should contain enzyme systems capable of cleaving ACTH to generate CLIP and a tridecapeptide, and of amidating and acetylating the latter. Preliminary observations indicate that rat posterior lobe extracts contain an enzyme which breaks down ACTH in the 13 to 18 part of the molecule, leaving C-terminal immunoreactivity intact. The identification of CLIP in a human tumour associated with the actopic syndrome suggests the presence of similar enzyme systems in human tumours, and Orth (1973) has reported that tumour extracts contain a peptide resembling CLIP together with equivalent amounts of an MSH-active peptide with a molecular weight higher than α-MSH (approx. 16 to 17 amino acids). This peptide cross-reacted with an N-terminally directed ACTH antiserum,

but not with an α-MSH antiserum. There is thus the possibility that this peptide is the 1 to 16 or 1 to 17 fragment of the ACTH molecule, and it would suggest that tumours have the ability to cleave ACTH, as in the pars intermedia cells, but not to modify the N-terminal fragment to α-MSH.

Finally, there should be phylogenetic evidence for the presence of a α-MSH and CLIP. Strong support for the present hypothesis comes from recent studies on the structure of the ACTH-like peptides of the dogfish Squalus acanthias. α-MSH has previously been isolated and characterised from neurointermediate lobe extracts of this species (Lowry & Chadwick, 1970a). More recently, (Bennett, Lowry, McMartin and Scott, unpublished observations) ACTH has been isolated from rostral lobe extracts, and characterisation has revealed that it is thirty-nine amino acids in length and has an N-terminal 1 to 13 sequence identical to that of mammalian ACTH except for a methionine-for-valine replacement at position 13. This replacement is found in the dogfish α-MSH. Studies of the neurointermediate lobe extracts resulted in the isolation and characterisation of a peptide identical in sequence to the 18 to 39 portion of dogfish ACTH (i.e. CLIP). There are eight amino acid differences between the α^{18-39} portions of dogfish and porcine ACTH, and these exact differences are found in the corresponding CLIP molecules. A separate genetic origin for ACTH and CLIP would seem very unlikely in the light of this evidence.

The stage in evolution at which the proposed cleavage mechanism emerged cannot yet be assessed precisely. There is evidence however, that MSH activity antedates ACTH activity, since lamprey pituitaries contain MSH in

significant amounts but not corticotrophin (see above). It is possible that the presumptive ACTH molecule is present in the cyclostomes as a pro- α-MSH molecule. This may later in evolution have found a role in stimulating adrenal cells. Independent formation and release of these hormones might then depend upon the differentiation of the pituitary gland into a part concerned with the release of intact ACTH (viz. the pars distalis) and one in which ACTH is cleaved (viz. the pars intermedia). At a time during evolution when α-MSH and ACTH were both important to survival, amino acid substitutions within the 1 to 13 sequence would have been subject to natural selection for both activities. Since it is likely that few mutations would be advantageous for both these activities, this may explain the constancy of α-MSH (Lowry & Chadwick, 1970b; Shapiro et al., 1972) and the amino-terminal part of ACTH during evolution.

Mechanism of cleavage

The present hypothesis envisages that ACTH synthesised in the intermediate lobe is a precursor of α-MSH and CLIP, and the formation of the two latter peptides is controlled initially by intracellular proteolysis. This mechanism resembles that demonstrated or proposed for the formation of other active substances from their precursor forms. Thus the conversion of 'big gastrin' (Gregory & Tracy, 1972) to its active form, and proinsulin to insulin (Steiner et al., 1969; Sando et al., 1972) can be achieved by trypsin treatment. Several non-hormonal proteins have been shown to be activated by a very similar mechanism involving the partial proteolytic digestion of a precursor, resulting in the formation of a smaller active product e.g.

blood clotting proteins, gut enzymes (Ottensen, 1967).

It has been suggested that the lipotrophic hormones (β-LPH and -LPH) isolated from ovine, porcine and human pituitaries are precursors of β-MSH, since the complete sequences of the β-MSHs from these three species are present in the 41 to 58 portions of the homologous LPHs (37 to 58 for human β-MSH). The localisation of α-MSH and β-MSH in the pars intermedia of the pig and sheep pituitary (Dubois, 1972) suggests that the cleavage of LPH to β-MSH, and of ACTH to α-MSH takes place in the same cell. Cleavage of LPH should yield additional peptides (analogous to CLIP) in the pars intermedia which have yet to be identified. By analogy with the ACTH-CLIP system, intact β-LPH should be found in the corticotrophs of the pars distalis.

At the proposed sites of cleavage of proinsulin, β-LPH and ACTH a characteristic sequence of two or more basic amino acids occurs (Fig. 26). This arrangement suggests that these regions may be more prone to cleavage by the specific enzyme involved than single basic residues. This would be of considerable functional importance, preventing the enzymes from further splitting the active peptide into inactive fragments, as would occur if there were only single basic amino acids present. It also suggests that the arrangement of basic residues in ACTH is not solely concerned with the activity of the molecule (i.e. binding the molecule to the adrenal receptor) but also exists for the formation of α-MSH and CLIP.

<u>Role of CLIP</u>

So far CLIP has been considered as of secondary importance in the formation of α-MSH from ACTH (i.e. it represents a

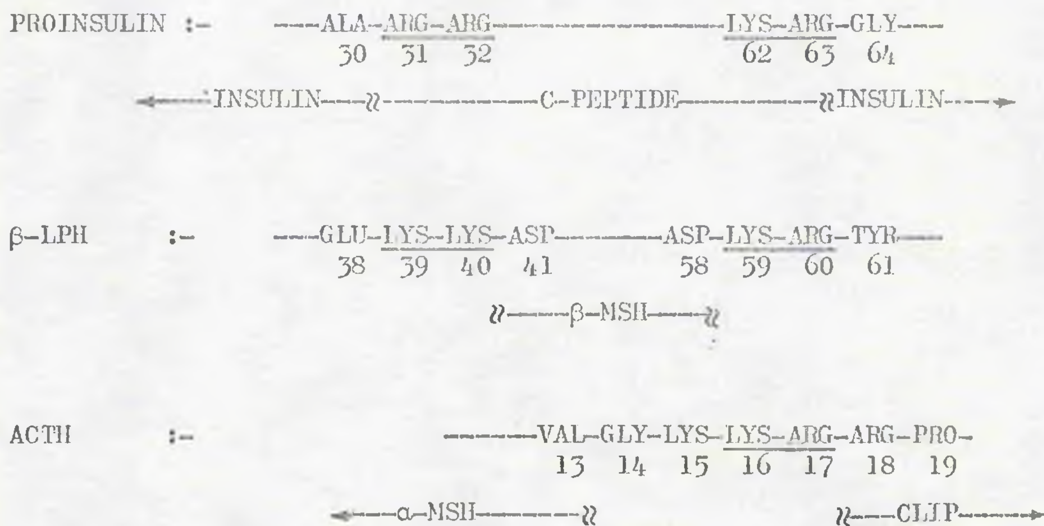


Fig 26 : Amino acid sequences in the regions of the proposed sites of enzymatic cleavage of proinsulin , β- lipotrophin and ACTH , demonstrating the occurrence of pairs of basic amino acids.

discarded fragment). There is no reason for discounting the possibility, however, that CLIP may possess a biological action.

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APPENDIX 1

POSSIBLE ROLES OF THE MELANOCYTE-STIMULATING HORMONES IN MAMMALS

1) α-MSH :-

- delays the extinction of conditioned avoidance responses in rats (Bohus, 1971)
- stimulates the mouse thyroid (Bowers et al., 1964)
- causes marked natiuresis in the rat (Orias & McCann, 1972)
- stimulates rat preputial gland growth (Krakenbuhl & Desaulles, 1969)

2) β-MSH :-

- injected intravenously into cats, results in increased monosynaptic potentials in the spinal cord (possible C.N.S. role) (Krivoy & Guillemin, 1961)
- injected intracisternally into dogs, results in stretching and yawning reflex (Ferrari et al., 1961)
- causes aqueous flare in the eyes of the rabbit (Dyster-Aas & Krakau, 1965)
- stimulates the mouse thyroid (Bowers et al., 1964)
- has equivalent steroidogenic potency to ACTH in the rabbit (Rudman et al., 1970)
- causes natiuresis in the rat (Orias & McCann, 1972)
- has a noticeable effect on several of the constituents of human serum (Kastin et al., 1968)

3) The pituitary MSH content and plasma MSH levels have been found to vary in the following situations:-

- in the female rat, copulation and vaginal stimulation depletes the pituitary MSH store (Taleisnik & Tomatis, 1968), as does suckling (Taleisnik & Orias, 1966)
- in castrated male spanish goats, there is a correlation between temperature and plasma MSH levels (Peaslee & Millburn, 1971).

APPENDIX 2

CORTICOTROPHIN IN THE NON-MAMMALIAN VERTEBRATES - PUBLISHED COMPARATIVE STUDIES

a) EFFECTS OF HYPOPHYSECTOMY:-

1) Species in which it has been noted that hypophysectomy leads to adrenal atrophy:-

ELASMOBRANCHS - Torpedo torpedo (Dittus, In Hoar, 1966)

TELEOSTS - Anguilla sp. (eel) (Oliverau, 1965; Hanke et al., 1967; Butler et al., 1969). Carassius auratus (goldfish) (Chavin & Kovacevic, 1961). Couesius plumbeus (van Overbeeke & Ahsan, 1966). Tilapia mossambica (Basu et al., 1965)

AMPHIBIANS - Rana sp. (frogs - summer season) (Chester Jones, 1957; van Kemenade, 1968). Bufo arenarum (toad) (Houssay, 1949). Triturus torosa (newt) (Miller, 1953).

REPTILES - Thamnophis sp. (snake), Xantusia vigilis (lizard), Agama sp.(lizard) (In Chester Jones, 1957). Dipsosaurus dorsalis (Chan et al., 1970).

2) Species in which it has been noted that hypophysectomy leads to a fall in plasma corticosteroid levels:-

TELEOSTS - Anguilla sp. (Butler et al., 1969; Donaldson & McBride, 1967). Poecilia latipinna (Hawkins et al., 1970)

AMPHIBIANS - Rana catesbiana (Johnston et al., 1967)

REPTILES - Anolis carolinensis (Licht & Bradshaw, 1969)

BIRDS - Gallus sp. (chicken) (Nagra et al., 1963 - only 40% fall)

3) Species in which it has been noted that hypophysectomy fails to lead to adrenal atrophy:-

CYCLOSTOMES - Lampetra fluviatilis (Hardisty, 1972)

ELASMOBRANCHS - Scyliorhinus caniculatus (Dodd, In Hoar, 1966)

TELEOSTS - Fundulus heteroclitus(Pickford, In Pickford & Atz, 1957)

AMPHIBIANS - *Rana* sp. (frogs - winter season)(Chester Jones, 1957)

BIRDS - *Gallus* sp., *Columber* sp., *Anas* sp.(see Miahle & Koch, 1969)

4) Species in which it has been noted that hypophysectomy fails to cause a fall in plasma corticosteroid levels:- None

b) EFFECT OF INJECTING MAMMALIAN ACTH:-

1) Species in which it has been noted that mammalian ACTH will restore adrenal atrophy in hypophysectomised animals or cause adrenal hypertrophy in normal animals:-

ELASMOBRANCHS - *Torpedo torpedo* (Dittus, In Hoar, 1966)

TELEOSTS - *Carassius auratus*, *Conger conger*, *Fundulus heteroclitus*, *Astyanax mexicanus*, *Anguilla anguilla* (see Pickford & Atz, 1957). *Anguilla anguilla*(Hanke *et al.*, 1967). *Tilapia mossambica* (Basu *et al.*, 1965)

AMPHIBIANS - *Rana temporaria* (Hanke & Weber, 1965; van Kemenade, 1968). *Triturus torosa* (Miller, 1953)

REPTILES - *Natrix natrix*, *Xantusia vigilis* (see Chester Jones, 1957). *Dipsosaurus dorsalis* (Chan *et al.*, 1970)

BIRDS - *Gallus* sp. (Zarrow *et al.*, 1962)

2) Species in which it has been noted that injection of mammalian ACTH will cause a rise in plasma corticosteroid levels in hypophysectomised or normal animals:-

TELEOSTS - *Onchorhynchus tshawytscha* (salmon) (Hane *et al.*, 1966). *Anguilla* sp. (Leloup-Hatey, 1964 - in Miahle & Koch, 1969; Bradshaw & Fontaine-Bertrand, 1968; Butler *et al*, 1969). *Poecilia latipinna* (Hawkins *et al.*, 1970)

AMPHIBIANS - *Rana catesbiana* (Johnston *et al.*, 1967)

REPTILES - *Anolis carolinensis* (Licht & Bradshaw, 1969)

BIRDS - *Gallus* sp. (Nagra *et al.*, 1963)

3) Species in which it has been noted that injection of mammalian ACTH fails to restore adrenal atrophy in hypophysectomised animals:-

CYCLOSTOMES - Lampetra fluviatilis (Hardisty, 1972)

TELEOSTS - Couesius plumbeus (van Overbeeke & Ahsan, 1966)

REPTILES - Agama stellio (Frenkel & Kraicer, 1971)

4) Species in which it has been noted that ACTH fails to cause a rise in plasma corticosteroid levels in hypophysectomised or normal animals:-

TELEOSTS - Salmo gairdnerii (Hill & Fromm, 1968)

REPTILES - Pseudemys sp. (turtle), Caiman sclerops (alligator) (Nothstine et al., 1971)

c) EFFECT OF INJECTING VERTEBRATE PITUITARY EXTRACTS INTO MAMMALIAN RECIPIENTS

1) Reports in which vertebrate pituitary extracts stimulated one or more parameters of mammalian adrenal function such as ascorbic acid depletion in the rat adrenal (AAD) - employing the assay described by Sayers and his colleagues (1948); weight maintenance of the rat adrenal (AW) or adrenal corticosteroidogenis by adrenals of the mouse or rat (AC). A variety of in vitro and in vivo assay procedures have been employed for the latter studies.

SPECIES	ASSAY	REFERENCE
Myxine glutinosa hagfish	AAD Rat	Mowell & Phillips (1962)
Myxine glutinosa "	AW Mouse	Strahan(1959,1962)
Lampetra fluviatilis lamprey	AW Mouse	" " "
Katsuwonus vagans	AAD Rat	Ito et al.,(1952)
Thynnus orientalis tunny	AAD Rat	" "
Oncorhynchus keta salmon	AAD Rat	Rinfret & Hane(1955)
Gadus morhua cod	AAD Rat	Woodhead (1960)

SPECIES	ASSAY	REFERENCE
Carassius auratus goldfish	AC Mouse (in vitro)	Purrott & Sage(1967) Sage & Purrott(1969)
Cyprinus carpio carp	AC Mouse (in vivo)	Fontaine-Bertrand et al., (1969)
Salmo salar salmon	AC Mouse (in vivo)	" "
Rana pipiens frog	AC Rat (in vitro)	Preslock & Brinkley (1970)
Rana catesbiana bullfrog	AC Rat (in vivo)	Shapiro et al.,(1972)
Gallus gallus chicken	AC Rat (in vivo)	" " "

2) Reports in which vertebrate pituitary extracts failed to stimulate mammalian adrenal function:-

Pseudopleuronectes americans flounder	AC Rat (in vivo)	Shapiro et al.,(1972)
Iguana iguana iguana	AC Rat (in vivo)	" " "

d) EFFECT OF VERTEBRATE PITUITARY EXTRACTS INJECTED INTO NON-MAMMALIAN RECIPIENTS

1) Reports in which vertebrate pituitary extracts stimulated adrenal function in non-mammalian recipients:-

DONOR.	RECIPIENT	EFFECT	REFERENCE
ELASMOBRANCHS			
Squalus acanthus			
Raja rhina	Chicken adrenals	Increased c-steroid	de Roos & de Roos
Hydrolagus colliei	(in vitro)	production	(1967)
TELEOSTS			
Cyprinus carpio	Hypox. eels	Increased plasma c-steroid levels	Bradshaw & Fontaine-Bertrand (1968)
Oncorhynchus t.	Hypox. Couesius plumbeus.	Reversed adrenal atrophy	Van Overbeek & Ahsan(1966)

AMPHIBIANS			
Rana catesbiana	Homologous adrenals	Increased c-steroid production	Cartensen *et al.* (1961)
REPTILES			
Alligator	Chicken adrenals (*in vitro*)	"	Gist & de Roos (1966)
Naja naja	Homologous adrenals	"	Huang & Phillips (1967)
BIRDS			
Gallus sp.	"	"	de Roos & de Roos (1964)

APPENDIX 3

LOCALISATION OF ACTH AND MSH ACTIVITY IN VERTEBRATE PITUITARIES

	ACTH	MSH
CYCLOSTOMES	?	Removal of lamprey _meta-adenohypophysis_ leads to permanent skin pallor (Young, 1935)
ELASMOBRANCHS	Bulk of bioactive ACTH is present in _rostral lobe of pars distalis_ of three species (de Roos & de Roos, 1967)	Neurointermediate lobe of all species studied is rich in MSH activity. (Pickford & Atz, 1957)
TELEOSTS	Bulk of bioactive ACTH is present in _anterior_ portion of salmon pituitary (Fontaine-Bertrand _et al._, 1969)	_Posterior_ portion of the salmon pituitary contains bulk of MSH bioactivity (Fontaine-Bertrand _et al._, 1969)
	Injection of metopirone in two teleost species causes hypertrophy of _pro-adenohypophyseal_ cells which border the invading neurohypophysis (Ball & Oliverau, 1966)	_Posterior_ halves of trout pituitaries release most MSH activity in organ culture (Baker, 1967)
	Similar cells in the salmon _pro-adenohypophysis_ can be detected immunohistologically with anti-ACTH sera (McKeown & van Overbeeke, 1970).	
AMPHIBIANS	Bulk of bioactive ACTH is present in _pars distalis_ in frog (Preslock & Brinkley, 1970)	Skin darkening of tadpoles is produced with adult _pars intermedia_ extracts (Allen, 1920)
	ACTH activity corresponds to a region of basophilic cells in _rostral_ portion of _pars distalis_ in the frog and clawed toad (Larsen _et al._, 1971). These cells, in the _pars distalis_, hypertrophy in response to metopirone (van Kemenade, 1969)	
	Acidophilic cells in the _pars distalis_ of urodeles immunostain with anti-ACTH serum (Doerr-Schott & Dubois, 1970)	