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Isolation of the C-Fragment and C′-Fragment of Lipotropin from Pig Pituitary and C-Fragment from Brain

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Three novel peptides derived from lipotropin, the C-Fragment (residues 61–91), C′-Fragment (61–87) and N-Fragment (1–38), were isolated from pig pituitary, and the C-Fragment was shown to be present in brain. The experimental procedures developed for their isolation are described. The formation of each of the fragments involves enzymic cleavage of lipotropin at consecutive basic residues, with specificity identical with that involved in the activation of known prohormones. In brain assays C-Fragment exhibits a range of biological activities related to its ability to act as an inhibitory neurotransmitter.

During the course of a study on prohormones and hormones in pig pituitary, a series of previously unknown peptides was isolated and characterized (Bradbury *et al.*, 1975, 1976*a*). Two of these, one of which was a glycopeptide, were assigned to the *N*-terminal region of a polypeptide which was predicted to be the prohormone of corticotropin (ACTH); the remaining three were identified as fragments of lipotropin, a 91-residue peptide with an unknown function. With the accumulation of evidence that corticotropin is biosynthesized from a prohormone with a glycopeptide in its *N*-terminal region (Eipper & Mains, 1975) and that procorticotropin is elaborated contiguously with lipotropin (Mains *et al.*, 1977; Pelletier *et al.*, 1977) it is becoming clear that the series of peptides we isolated from the pituitary account for most of the structure of this multiple prohormone (Smyth, 1978).

In the present paper we describe the isolation of three fragments of the lipotropin molecule, comprising residues 1–38, 61–87 and 61–91. Their identification was based on amino acid composition together with *N*- and *C*-terminal analysis. These peptides have been found to be present in substantial quantity in pig pituitary glands and in a preliminary report were named N-Fragment, C′-Fragment and C-Fragment respectively (Bradbury *et al.*, 1976*b*). Further experiments have shown that mild digestion of lipotropin with trypsin or a pituitary enzyme leads to formation of the C-Fragment, but even extended digestion with trypsin fails to release β-melanotropin (β-MSH, lipotropin residues 41–58). Since many intracellular peptide hormones are generated by mild tryptic digestion of the corresponding prohormone, the results indicated that C-Fragment might be a functionally important region of lipotropin.

Concurrent studies on pituitary and brain were providing evidence for the existence of endogenous peptides with morphine-like properties (Terenius & Wahlström, 1974; Cox *et al.*, 1975; Hughes, 1975; Pasternak *et al.*, 1975). The molecular weight of the pituitary peptide was reported to be approximately 2000, corresponding to about 18 residues, whereas the brain peptides appeared to be smaller. The first opiate-active peptide to be identified was a pentapeptide, which was obtained in small quantity from brain (Hughes *et al.*, 1975); named [methionine]-enkephalin, it proved to correspond to the first five residues of lipotropin C-Fragment.

Comparison of the binding properties of C-Fragment (lipotropin residues 61–91), C′-Fragment (residues 61–87) and [methionine]enkephalin (residues 61–65) for brain opiate receptors showed that the affinity of the 31-residue peptide was much greater than the affinity of the shorter peptides (Bradbury *et al.*, 1976*c*). Furthermore it was found that C-Fragment is unique in its potency as an analgesic peptide (Feldberg & Smyth, 1976, 1977*a*,*b*; Bradbury *et al.*, 1977; Van Ree *et al.*, 1976; Loh *et al.*, 1976*a*) and possesses a range of other properties (Gispen *et al.*, 1976; Gent *et al.*, 1977; Loh *et al.*, 1976*b*). We describe here the detailed experimental procedures developed for the isolation of N-Fragment, C-Fragment and C′-Fragment in high yield from pig pituitary and evidence is given that C-Fragment is present also in brain.

Materials and Methods

Materials

Pig pituitaries were obtained from Walls Meat Co., Hyde, Cheshire, U.K. The pituitaries were removed

within 20min of slaughter, frozen in liquid N_2 and stored at −40°C. Trypsin (205units/mg) and chymotrypsin (47units/mg) were from Worthington Biochemical Corp., Freehold, NJ, U.S.A. *Armillaria mellea* proteinase was generously given by Dr. H. Gregory (I.C.I., Alderley Park, Cheshire, U.K.). Sephadex products were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Methods

Extraction of pituitaries. The procedure leading to the isolation of the N-, C- and C′-Fragments of lipotropin commenced with the homogenization of pituitaries and preparation of salt precipitates by a modification of the method of Li *et al.* (1965). The chromatographic isolation of peptides is novel.

Whole pig pituitaries (approx. 1200, 500g) were homogenized at 4°C in 2500ml of acid/acetone (water/acetone/conc. HCl, 5:40:1, by vol.) by using a Waring blender, and the suspension was stirred for 2h at room temperature. The mixture was centrifuged at 1000*g* for 20min and the pellet further extracted with 1 litre of 80% (v/v) acetone. The second extract was centrifuged for 30min, the two supernatants were combined and ^{125}I-labelled C-Fragment (10^6 c.p.m.; approx. 0.1nmol), prepared by the method of Hunter & Greenwood (1962), was added to aid the isolation of C-Fragment. The solution was poured into 19 litres of cold acetone. The precipitated solid (16.5g) was isolated by filtration, taken up in 700ml of water and the pH adjusted to 3 by addition of 4ml of 1M-NaOH. The solution was stirred during the addition of 45ml of saturated NaCl. After centrifugation at 1000*g* for 45min, the supernatant was removed and a second precipitation carried out by saturation with NaCl. Centrifugation at 1000*g* for 45min provided a pellet which formed the starting material for chromatographic isolation of the lipotropin fragments.

Resolution of peptides by gel filtration and ion-exchange chromatography. Peptides were fractionated in Pyrex glass columns fitted with Whatman discs. Ion-exchange resins were suspended in the appropriate equilibrating buffer and the pH was adjusted to the required value before packing into columns. The eluates were collected in fractions which were monitored for A_{230} or A_{277} to allow detection of the separated peptides.

Amino acid analysis. Samples were submitted to hydrolysis in redistilled 6M-HCl in sealed evacuated tubes at 100°C for 16h and analysis was carried out by the procedure of Spackman *et al.* (1958) by using a Beckman 120C amino acid analyser. The recovery of tyrosine and methionine was increased by addition of a crystal of phenol to the hydrolysis mixtures and by thorough de-aeration before sealing.

Purity of isolated peptides. Peptides obtained by ion-exchange chromatography were identified and characterized principally by amino acid analysis. In some cases *N*- and *C*-terminal analysis was carried out by using the cyanate end-group procedure (Stark & Smyth, 1963) and digestion with carboxypeptidase enzymes (Ambler, 1967). Peptides were further characterized by isolation of the fragments produced by enzymic digestion.

Phosphate buffers. Sodium phosphate buffers at pH values between 6 and 9 were prepared by addition of conc. HCl to Na_2HPO_4 at the required concentration. Phosphate buffers at pH4–6 were prepared from NaH_2PO_4.

Experimental and Results

The pellet obtained by extraction of the pituitaries was partially dissolved in 75ml of 0.1M-acetic acid. After centrifugation at 1000*g* for 30min, the residual pellet was dissolved in a further 75ml of 1M-acetic acid. The two extracts were treated separately during subsequent purification and are referred to in the text as extracts a and b. Each solution was desalted on Sephadex G-25 (column dimensions 60cm × 9cm) in 0.1M-acetic acid (elution volume 700–880ml) and chromatographed on a column (40cm × 4cm) of CM-Sephadex C-25 equilibrated with 0.02M-ammonium acetate, pH4.6. Stepwise elution was performed first with the pH4.6 buffer, then with 0.2M-ammonium acetate, pH6.8, and finally with 1M-ammonium acetate, pH6.8 (Figs. 1*a* and 1*b*). In each case essentially all the radioactive peptide was present in the final eluate.

Isolation of N-Fragment (lipotropin residues 1–38)

The peptides eluted from the CM-Sephadex columns by 0.02M-ammonium acetate, pH4.6, were combined and desalted on Sephadex G-25 (column dimensions 40cm × 2.5cm) in 0.1M-acetic acid. The desalted material was chromatographed on DEAE-Sephadex A-25 (column dimensions 100cm × 1cm) in 0.02M-sodium phosphate, pH7.0. The peptides were resolved with a linear gradient (total volume 400ml) of 0–2M-NaCl in the phosphate buffer (Fig. 2). The eluate from 373 to 416ml contained the octatridecapeptide (3.1μmol) accounting for residues 1–38 of lipotropin (Table 1).

Isolation of lipotropin (residues 1–91), γ-lipotropin (1–58), C′-Fragment (61–87) and β-melanotropin (41–58)

The peptides eluted from the CM-Sephadex column by 0.2M-ammonium acetate, pH6.8, were desalted on Sephadex G-25 (column dimensions 45cm × 3cm) in 0.1M-acetic acid, and further gel

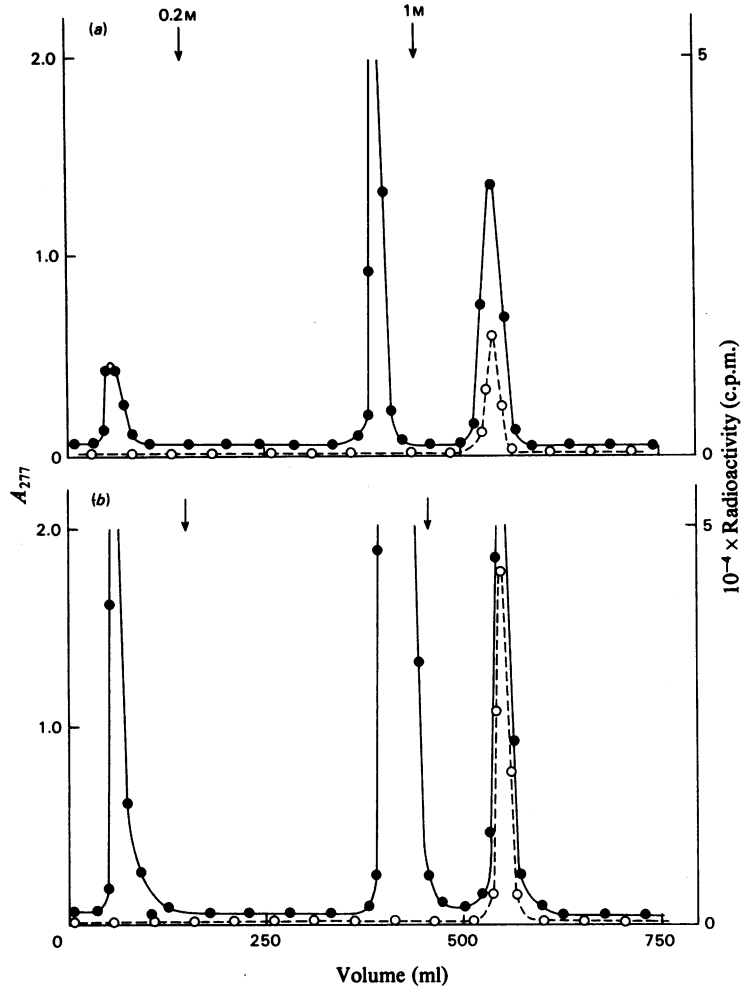

Fig. 1. *Chromatography of pituitary peptides on CM-Sephadex C-25 after (a) first extraction and (b) second extraction of pituitary pellets*

For details see the text. ○, Radioactivity; ●, A_{277}.

filtration was carried out on Sephadex G-50 (column dimensions 150cm × 2cm) in 0.02M-sodium phosphate, pH7.0. The elution profiles for extracts a and b are shown in Figs. 3(*a*) and 3(*b*). The peptides in peak A1 were desalted on Sephadex G-25 (40cm × 1.5cm) and chromatography was carried out on DEAE-Sephadex A-25 (column dimensions 65cm × 1cm) eluted with 0.01M-sodium phosphate at pH8.0, by using a linear gradient of 0–0.5M-NaCl (total volume 400ml) in the phosphate buffer. Fractions corresponding to the major peak (elution volumes 66–110ml) were combined and shown by analysis (Table 1) to contain homogeneous lipotropin (12.3μmol). γ-Lipotropin was obtained from extracts a and b by chromatography of the peptide mixture of peak A2 (Figs. 3*a* and 3*b*) on DEAE-Sephadex A-25 (column dimensions 120cm × 1cm) in 0.02M-sodium phosphate, pH7.0, by using a linear gradient of 0–1M-NaCl (total volume 400ml). The fraction 138–160ml was shown to contain 110mg (11.3μmol) of γ-lipotropin. The peptides in peak A3 were chromatographed on SP (sulphopropyl)-Sephadex C-25 (column dimensions 70cm × 0.9cm) with a linear gradient (volume 400ml) of 0–0.5M-NaCl in 0.02M-sodium phosphate, pH7.0 (Fig. 4) to give C'-Fragment (5.0μmol) as the main product. Homogeneity was established by analysis (Table 1) and by digestion with exopeptidases. Peak A4 from extract (a) proved to be homogeneous β-melanotropin (lipotropin residues 41–58, 0.4μmol).

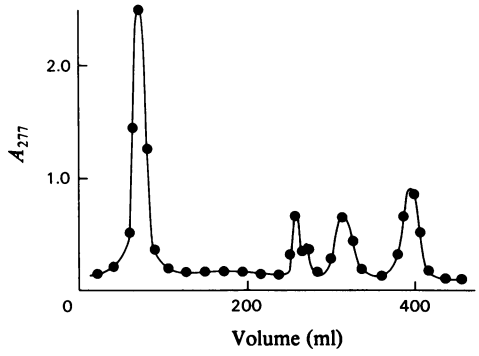

Fig. 2. *Isolation of the N-Fragment of lipotropin on DEAE-Sephadex A-25*
For details see the text.

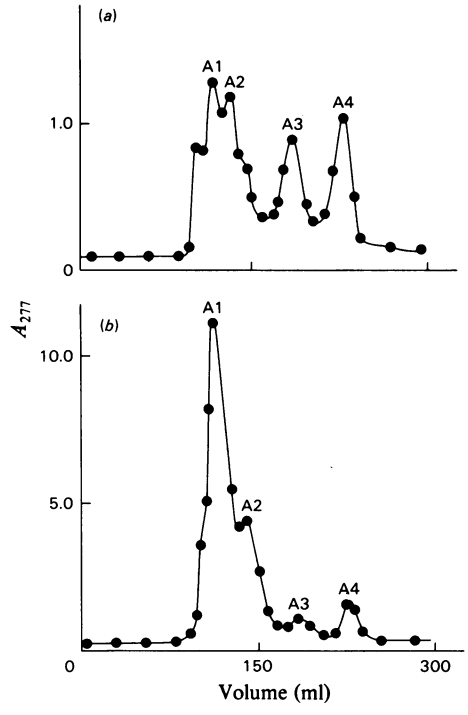

Fig. 3. *Gel filtration of material obtained from the* 0.2M *eluate of the CM-Sephadex C-25 column as shown in Fig.* 1
The chromatogram shown in (*a*) refers to peptides obtained by mild extraction of the crude pituitary pellet, as in Fig. 1(*a*). The chromatogram (*b*) refers to the peptides obtained by dissolving the residual pellets (Fig. 1*b*). The peaks A1, A2, A3 and A4 were studied in greater detail as described in the text.

Isolation of C-Fragment (lipotropin residues 61–91)

The peptides from extracts a and b eluted from CM-Sephadex in 1M-ammonium acetate (Fig. 1) were desalted on Sephadex G-25 (column dimensions 40cm × 3cm) in 50% (v/v) acetic acid, and after removal of acetic acid by concentration *in vacuo* the mixtures were chromatographed separately on a column (40cm × 1cm) of SP-Sephadex C-25 in 0.02M-sodium phosphate, pH7.0, with a linear gradient (400ml) of 0–0.5M-NaCl. The elution profiles are shown in Figs. 5(*a*) and 5(*b*). C-Fragment (total 9.1 μmol) was present in the fractions between 250 and 290ml, emerging in advance of the radioiodinated peptide. Homogeneity was confirmed by analysis (Table 1); the peptide was devoid of arginine and there was no evidence of a methionine–leucine replacement.

Preparation of peptide with lipotropin residues 61–69

C-Fragment (1 μmol) was digested with trypsin (60 μg) for 18h at 37°C in 1ml of 0.05M-sodium phosphate, pH8. The solution was acidified to pH2 by addition of HCl and chromatography was carried out on a column (30cm × 1cm) of Dowex 50 (X2) in 0.02M-pyridine acetate, pH4.5, at 50°C, with a linear gradient (90ml mixer) to 0.2M-pyridine. The tryptic peptide (0.91 μmol) corresponding to residues 61–69 of lipotropin emerged between 200 and 216ml. The peptide was devoid of leucine and the methionine content was indicative of an integral residue (Table 1).

Preparation of peptide with lipotropin residues 61–68

C-Fragment (1 μmol) was digested with *Armillaria mellea* proteinase (60 μg) for 10h at 37°C in 1ml of 0.05M-sodium phosphate, pH8, and the reaction was terminated by acidification with HCl to pH2. The digest was chromatographed on a column (30cm × 1cm) of Dowex 50 (X2) in 0.01M-pyridine acetate, pH4.0, with a linear gradient (90ml mixer) to 0.2M-pyridine. The fractions containing the first major peak at 80–94ml were combined and the peptide (0.53 μmol) was shown to be pure by analysis.

Preparation of peptides containing lipotropin residues 61–78 *and* 79–91

C-Fragment (1 μmol), to which ^{125}I-labelled C-Fragment (approx. 10^6 c.p.m.) was added, was digested with chymotrypsin (1.5 μg) in 1ml of 0.01M-sodium phosphate, pH8, for 2h at 20°C. The solution was desalted on Sephadex G-25 (column dimensions 40cm × 1cm) in 50% acetic acid. The products were chromatographed on sulphopropyl-Sephadex C-25 (40cm × 0.9cm) in 0.02M-sodium

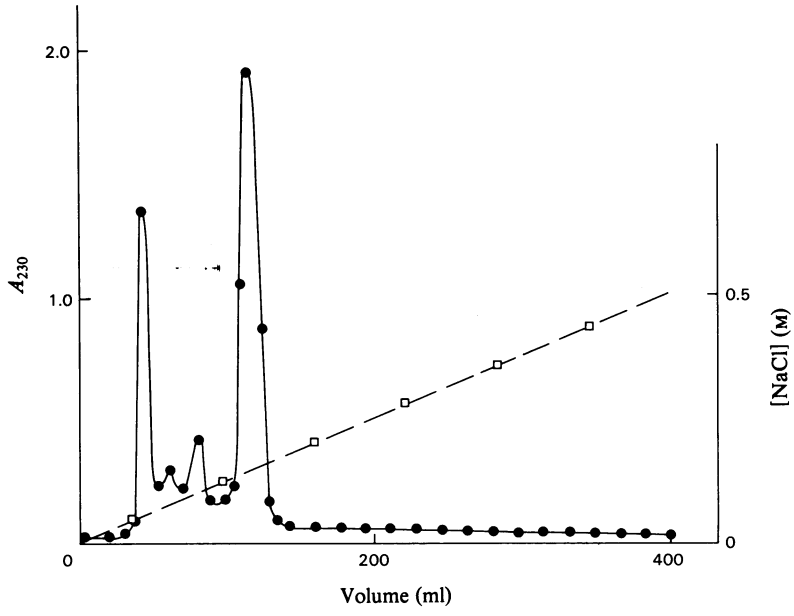

Fig. 4. *Chromatography of C'-Fragment (residues 61–87) on SP-Sephadex C-25*
The C'-Fragment was the peptide most strongly retained. ●, A_{230}; □, [NaCl].

Table 1. *Amino acid compositions of lipotropin fragments isolated from pig pituitary*
Peptides representing lipotropin residues 1–91, 1–58 (γ-lipotropin), 1–38 (N-Fragment), 41–58 (β-melanotropin), 61–87 (C'-Fragment) and 61–91 (C-Fragment) were extracted from pituitary; peptides representing residues 61–78, 61–69 and 61–68 were obtained by enzymic digestion of C-Fragment, as in the text. The numbers given show the composition of each peptide; values are expressed in molar residues (relative to the expected glycine content of lipotropin and its fragments) to the nearest one-tenth of a unit. Amino acids present in amounts <0.1 of a unit are omitted.

	Composition (no. of residues)								
Residue nos. ...	1–91	1–58	1–38	41–58	61–87	61–91	61–78	61–69	61–68
Lysine	10.0	3.9	—	1.9	2.4	4.8	1.1	1.0	—
Histidine	0.8	1.0	—	1.0	0.9	1.0	—	—	—
Arginine	3.9	3.0	—	1.0	—	—	—	—	—
Aspartic acid	5.2	2.6	1.3	1.8	1.8	2.1	—	—	—
Threonine	2.9	—	—	—	2.7	3.6	—	1.0	1.0
Serine	2.6	0.9	—	1.0	1.9	2.1	3.2	1.0	1.1
Glutamic acid	13.4	11.3	8.5	1.9	2.0	3.2	2.4	0.9	1.0
Proline	7.9	6.0	4.0	2.5	0.9	1.0	1.1	—	—
Glycine	8.0	5.0	3.0	2.0	2.0	3.0	2.0	2.0	2.0
Alanine	14.8	11.9	12.8	—	1.8	2.2	—	—	—
Valine	3.1	1.0	0.7	—	1.2	1.0	1.1	—	—
Methionine	1.6	0.9	—	0.9	0.8	0.8	1.0	0.4	0.7
Isoleucine	1.1	—	—	—	0.4	0.6	2.2	—	—
Leucine	5.9	3.2	2.7	—	1.8	1.9	2.2	—	—
Tyrosine	2.9	1.9	1.1	0.9	0.8	1.0	1.0	1.0	0.8
Phenylalanine	2.9	1.1	—	0.9	1.6	1.9	2.0	1.1	1.0

phosphate, pH7.0, by using a linear gradient of 0–0.5M-NaCl (Fig. 6). The radioactive fractions eluted between 55 and 82ml were combined and found to contain the homogeneous octadecapeptide (lipotropin residues 61–78, 0.83μmol); fractions emerging between 350 and 375ml contained the tridecapeptide (lipotropin residues 79–91, 0.63μmol) and were devoid of radioactivity.

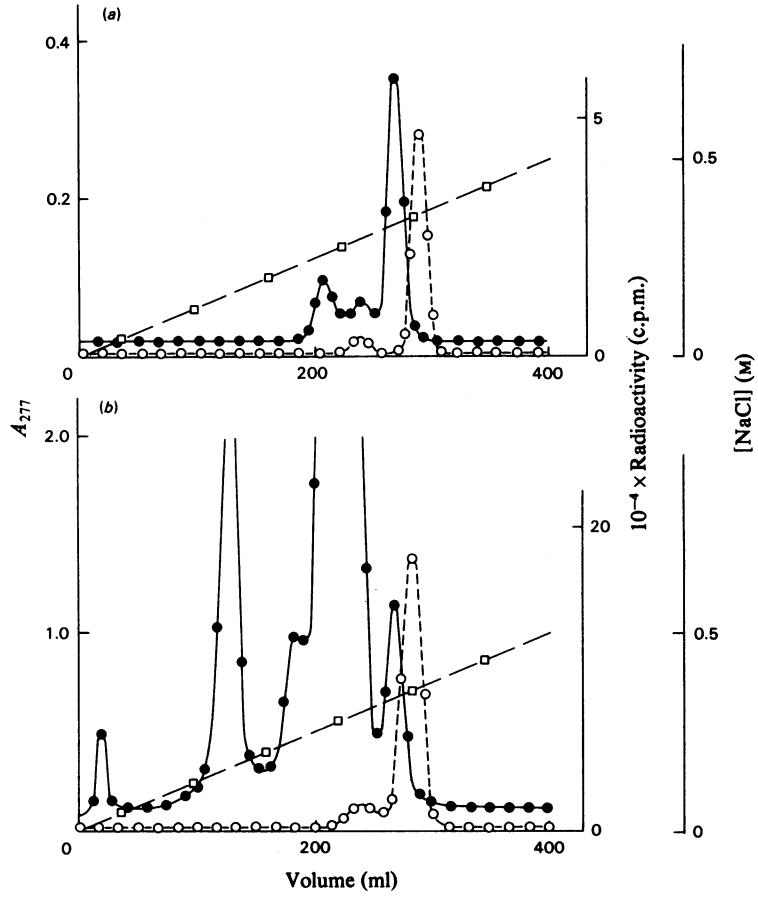

Fig. 5. *Chromatography of C-Fragment (residues 61–91) on SP-Sephadex C-25*
(*a*) and (*b*) correspond to peptides obtained by light and heavy extraction of pituitary pellet. C-Fragment is the peptide most strongly retained. ●, A_{277}; ○, radioactivity; □, [NaCl].

Isolation of C-Fragment from brain

Brain (1200g, obtained from 20 pigs), after excision of brain stem and cerebellum and with meticulous exclusion of pituitary, was homogenized in 2500ml of acid/acetone, with ^{125}I-labelled C-Fragment (approx. 10^6 c.p.m., 0.1nmol) added as a marker, and salt precipitates were obtained as described above for the isolation of C-Fragment from pituitary. The 1M-ammonium acetate fraction from CM-Sephadex was desalted in 50% acetic acid and chromatography was carried out on SP-Sephadex C-25 (Fig. 7). Fractions were monitored at 277nm and opiate-binding assays were performed (Bradbury *et al.*, 1976*c*), which revealed the presence of an opiate-active peptide equivalent to approx. 30pmol of C-Fragment/g of brain tissue. The peptide emerged in the known elution position of C-Fragment, immediately in advance of ^{125}I-labelled C-Fragment, which was added in trace quantity as a marker. Its pharmacological properties, assessed by displacement of [^{3}H]dihydromorphine and [^{3}H]naloxone from brain opiate receptors *in vitro*, were the same as those of the characterized C-Fragment isolated from pituitary (Birdsall *et al.*, 1976).

Discussion

The amounts of C-Fragment and C′-Fragment obtained per 500g of pig pituitary were 9.1μmol and 5.0μmol respectively, extraction of 1200 pituitaries yielding 30.6mg of C-Fragment and 15.1mg of C′-Fragment. Since the recovery of ^{125}I-labelled C-Fragment added as a marker to the homogenized tissue was 42%, the concentration of C-Fragment in the pituitary must be at least 43nmol/g of tissue. In contrast, the concentration of C-Fragment deter-

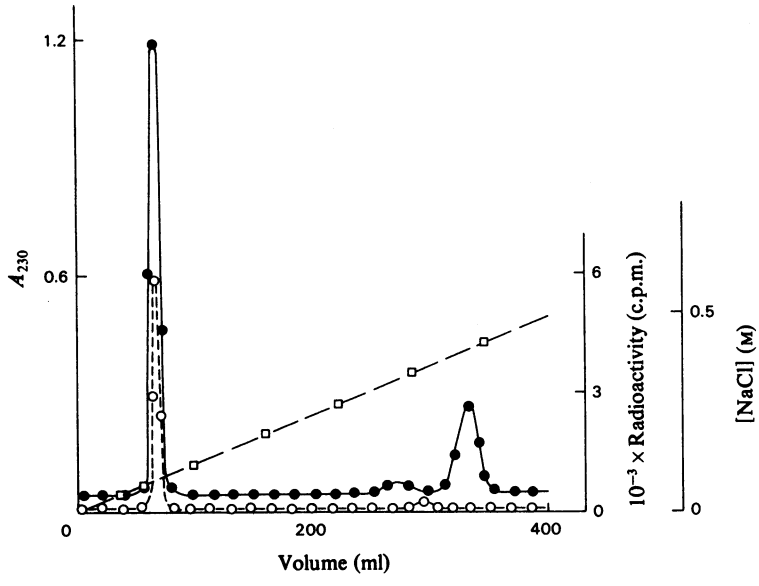

Fig. 6. *Isolation of peptides containing lipotropin residues* 61–78 *and* 79–91 *by chromatography of a chymotryptic digest of C-Fragment on SP-Sephadex C-25*
For details see the text. ●, A_{230}; ○, radioactivity; □, [NaCl].

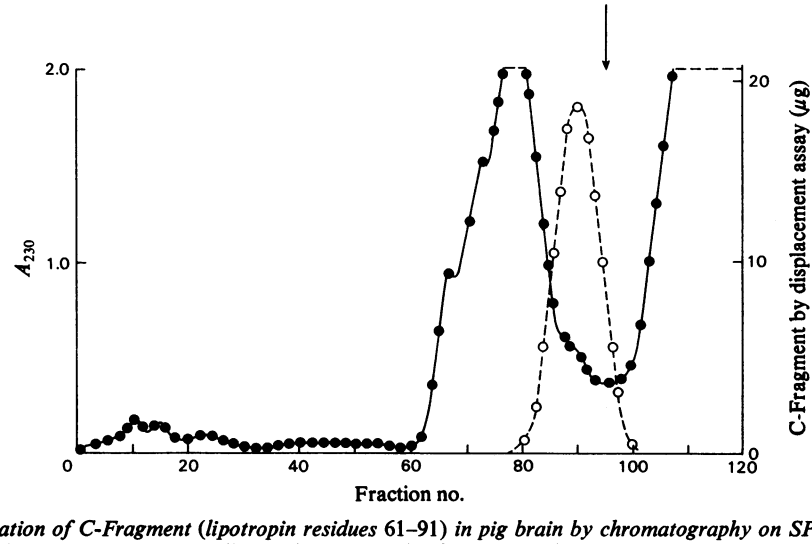

Fig. 7. *Identification of C-Fragment (lipotropin residues* 61–91) *in pig brain by chromatography on SP-Sephadex C-25*
For details see the text. ●, A_{230}; ○, radioactivity; □, affinity for brain opiate receptors as measured by the displacement of [^{3}H]naloxone from brain membrane. Fractions were of 3ml. The arrow shows the position of ^{125}I-labelled C-Fragment.

mined in pig brain is about 40pmol/g of tissue (Smyth & Zakarian, 1978).

C-Fragment was found to exhibit anomalous behaviour during gel filtration. Attempts to desalt the peptide on Sephadex G-25 in neutral solution led to poor recovery and little improvement was obtained at acid pH. The peptide was desalted successfully in 50% acetic acid and it behaved

normally during gel filtration in the presence of 8M-urea. The difficulty did not appear to arise during ion-exchange chromatography, but it may be relevant that high concentrations of salt were used in the displacement of the peptide from anion- and cation-exchange resins. It seems likely that the unusual chromatographic properties of C-Fragment are associated with its strongly basic character and high content of aromatic residues, but the peptide does not seem to aggregate. It was observed, however, that C-Fragment (31 residues) emerged before corticotropin (39 residues) during gel filtration on Sephadex G-50 in 0.01M-acetic acid. It was also observed that in preparing dilute solutions of C-Fragment for bioassay, absorption to glass took place, a difficulty that was overcome by the use of plastic tubes and pipettes, avoiding contact with glass or metal, and by including 1% serum albumin in the diluents.

From the compositions of the peptides isolated from pituitary it was clear that the release of N-Fragment, C-Fragment and C′-Fragment involves enzymic attack at consecutive basic residues of lipotropin, formation of N-Fragment involving cleavage in the region of lysyl-lysine at positions 39–40, C-Fragment cleavage in the region of lysyl-arginine at positions 59–60, and C′-Fragment at lysyl-lysine residues 88–89 (Fig. 8). The basic residues appear to be removed from the resulting fragments by enzymes with the specificity of carboxypeptidase B or aminopeptidase B.

None of the peptides isolated from pituitary was formed by cleavage at single basic residues. Thus the arginine–alanine bond at positions 23 and 24 in lipotropin and the arginine–tryptophan bond at positions 51 and 52 remained intact during the formation of γ-lipotropin, N-Fragment and β-melanotropin, yet mild digestion of lipotropin with trypsin leads to rapid cleavage at these positions (A. F. Bradbury & D. G. Smyth, unpublished work). It remains to be seen whether the pituitary enzyme involved in generating C-Fragment has an absolute requirement for paired basic residues or whether lipotropin and the 31K precursor possess a preferred conformation which renders the peptide bond at positions 60 and 61 unusually reactive. Studies of lipotropin by optical methods have indicated that in aqueous solution there is little ordered structure, but the molecule readily adopts conformation in a lipophilic environment (Bayley *et al.*, 1977; Hollosi *et al.*, 1977), which might exist within the secretory granules of the pituitary.

The high degree of specificity involved in the enzymic formation of C-Fragment and C′-Fragment implies that the peptides are the products of organized processes in the gland and are not formed by degrading enzymes, which would be relatively non-specific. It has been shown, too, that the C- and C′-Fragments are not artifacts of the isolation procedure: when rat pituitary was homogenized in acid/acetone in the presence of lipotropin, no detectable degradation took place (D. G. Smyth, unpublished work) and in the present experiments on the isolation of pituitary peptides from the pig there was no evidence of the formation of spurious products by autolysis *post mortem*. Furthermore, the high potency of C-Fragment in its biological properties, contrasting with the lack of activity exhibited by lipotropin (Feldberg & Smyth, 1977*a*), shows that the enzymes involved in its formation are activating enzymes.

The generation of C-Fragment and γ-lipotropin from lipotropin in the pituitary gland is formally equivalent to the formation of insulin and its con-

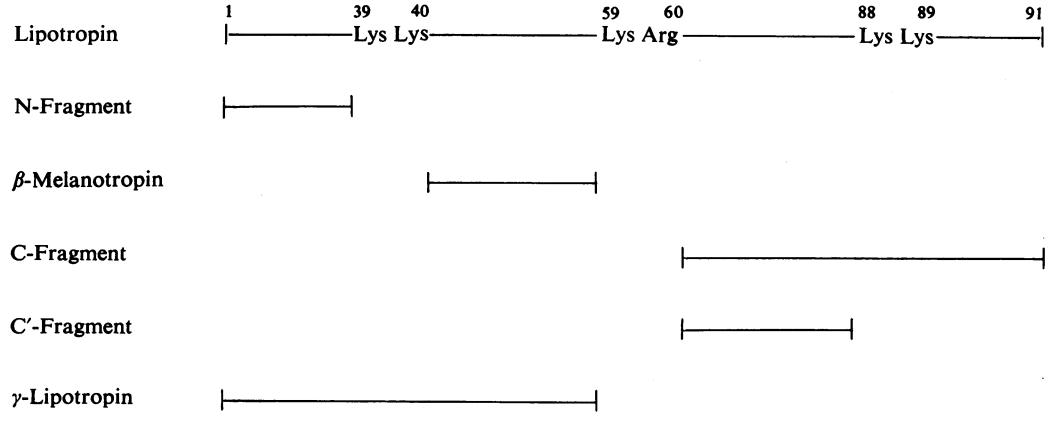

Fig. 8. *Fragments of lipotropin isolated from pig pituitary*

Table 2. *Biological actions of C-Fragment*

These pharmacological assays were carried out with C-Fragment isolated from pig pituitary as described in this paper. Precise evaluation of the potency of its inhibitory effect on neuronal firing requires determination of transport numbers.

Activity	Potency (relative to morphine = 1)	Reference
Analgesia	100	Feldberg & Smyth (1976, 1977*a*,*b*); Van Ree *et al.* (1976); Bradbury *et al.* (1977)
Inhibition of firing of isolated neurons	Active	Gent *et al.* (1977)
Hyperglycaemia	100	Feldberg & Smyth (1977*b*)
Stimulation of grooming behaviour	50	Gispen *et al.* (1976)
Inhibition of prostaglandin E_1-stimulated adenylase cyclase	60	H. O. J. Collier (unpublished work)

necting peptide from proinsulin in the β-cells of the pancreas. In that case, however, only small amounts of the prohormone remain in the gland, the major fraction being converted into insulin (Kemmler *et al.*, 1973). In the pituitary it appears that substantial amounts of lipotropin, together with the biologically active product C-Fragment, are present in the secretory granules. The biosynthetic mechanism involved in the formation of C-Fragment from lipotropin, and in turn from the multiple 31K prohormone, is obviously more complex than that involved in the elaboration of insulin.

The presence of C-Fragment in brain and its high potency in several assays (Table 2) indicates that it may fulfil an important role in the central nervous system. However, the function of C-Fragment elaborated in the pituitary (and of C'-Fragment) is not known. The normal amounts of C-Fragment in serum are low (S. Zakarian & D. G. Smyth, unpublished work), very much less than would be required to produce analgesia via the circulation, and it is not known whether a specific stimulus leads to increased secretion. It is possible that pituitary C-Fragment is the source of the C-Fragment in brain, but the anatomical connections between brain and pituitary have been considered to be unidirectional. Formation of C-Fragment in the pituitary followed by transference through a portal system or the cerebrospinal fluid to the brain would add complexity to control mechanisms. It is more likely, therefore, that C-Fragment is elaborated intracellularly in brain.

The availability of large quantities of homogeneous C-Fragment and C'-Fragment extracted from pituitary should open the way to investigation of the central and peripheral functions of these interesting peptides.

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References

Ambler, R. P. (1967) *Methods Enzymol.* **11**, 436–445

Bayley, P. B., Snell, C. R. & Smyth, D. G. (1977) *Biochem. Soc. Trans.* **5**, 683–686

Birdsall, N. J. M., Hulme, E. C., Bradbury, A. F., Smyth, D. G. & Snell, C. R. (1976) in *Proc. Int. Narcotics Res. Club Meet.: Opiates and Endogenous Opioid Peptides* (Kosterlitz, H. W., ed.), pp. 19–26, Elsevier–North-Holland, Amsterdam

Bradbury, A. F., Smyth, D. G. & Snell, C. R. (1975) in *Peptides: Chemistry, Structure and Biology* (Meienhofer, J. & Walter, R., eds.), pp. 609–615, Ann Arbor Science Publishers, Ann Arbor

Bradbury, A. F., Smyth, D. G. & Snell, C. R. (1976*a*) *Ciba Found. Symp.: Polypeptide Hormones: Molecular and Cellular Aspects* **41**, 61–75

Bradbury, A. F., Smyth, D. G. & Snell, C. R. (1976*b*) *Biochem. Biophys. Res. Commun.* **69**, 950–956

Bradbury, A. F., Smyth, D. G., Snell, C. R., Birdsall, N. J. M. & Hulme, E. C. (1976*c*) *Nature (London)* **260**, 793–795

Bradbury, A. F., Smyth, D. G., Snell, C. R., Deakin, J. F. W. & Wendlandt, S. (1977) *Biochem. Biophys. Res. Commun.* **74**, 748–754

Cox, B. M., Opheim, K. E., Teschemacher, H. & Goldstein, A. (1975) *Life Sci.* **16**, 1777–1782

Eipper, B. A. & Mains, R. E. (1975) *Biochemistry* **14**, 3836–3844

Feldberg, W. S. & Smyth, D. G. (1976) *J. Physiol. (London)* **260**, 30–31

Feldberg, W. S. & Smyth, D. G. (1977*a*) *Br. J. Pharmacol.* **60**, 445–454

Feldberg, W. S. & Smyth, D. G. (1977*b*) *J. Physiol. (London)* **265**, 25–27

Gent, J. P., Smyth, D. G., Snell, C. R. & Wolstencroft, J. H. (1977) *Br. J. Pharmacol.* **60**, 272

Gispen, W. H., Wiegant, V. M., Bradbury, A. F., Hulme, E. C., Smyth, D. G. & Snell, C. R. (1976) *Nature (London)* **264**, 794–795

Hollosi, M., Kajtan, M. & Gráf, L. (1977) *FEBS Lett.* **74**, 185–189

Hughes, J. (1975) *Brain Res.* **88**, 295–308

Hughes, J., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A. & Morris, H. R. (1975) *Nature (London)* **258**, 577–579

Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–499
Kemmler, W., Steiner, D. F. & Borg, J. (1973) *J. Biol. Chem.* **248**, 4544–4551
Li, C. H., Barnafi, L., Chretien, M. & Chung, D. (1965) *Nature (London)* **208**, 1093–1094
Loh, H. H., Tseng, L. F., Wei, E. & Li, C. H. (1976*a*) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2895–2898
Loh, H. H., Brase, D. A., Sampath Khanna, S., Mar, J. B., Way, E. L. & Li, C. H. (1976*b*) *Nature (London)* **264**, 567–568
Mains, R. E. Eipper, B. A. & Ling, N. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3014–3018
Pasternak, G. W., Goodman, R. & Snyder, S. H. (1975) *Life Sci.* **16**, 1765–1769
Pelletier, G., Leclerc, R., Labrie, F., Cote, J., Chrétien, M. & Lis, M. (1977) *Endocrinology* **100**, 770–776
Smyth, D. G. (1978) *Biochem. Soc. Trans.* **6**, 61–63
Smyth, D. G. & Zakarian, S. (1978) in *Proc. Symp. Endorphins in Mental Health* (Usdin, E., ed.), Macmillan Press, in the press
Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
Stark, G. R. & Smyth, D. G. (1963) *J. Biol. Chem.* **238**, 214–226
Terenius, L. & Wahlström, A. (1974) *Acta Pharmacol. Toxicol. Suppl.* **1**, 35
Van Ree, J. M., de Wied, D., Bradbury, A. F., Hulme, E. C., Smyth, D. G. & Snell, C. R. (1976) *Nature (London)* **264**, 792–794