Purification of a high-molecular-weight somatoliberin (growth-hormone-releasing factor) from pig hypothalami

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Preliminary observations [Sykes & Lowry (1980) *J. Endocrinol.* **85**, 42P–43P] had suggested that the major hypothalamic somatoliberin (growth-hormone-releasing factor) was a larger peptide than the other characterized hypothalamic factors, with an elution position on Sephadex G-50 between those of neurophysin and corticotropin. The present paper reports the isolation and preliminary characterization of pig hypothalamic somatoliberin. Acid extracts of pig stalk median eminence were purified by gel filtration and preparative and analytical high-pressure liquid chromatography to yield a preparation that was specific in the release of somatotropin (growth hormone) *in vitro*, giving a steep dose–response curve at doses in the range 0.20–3.0 ng. Amino acid analysis revealed a non-cysteine-containing peptide with a high number of glutamate (or glutamine) and aspartate (or asparagine) residues. The peptide had about 56–57 amino acid residues and an apparent molecular weight of 6400, in keeping with its elution position on a column of Sephadex G-50.

Several authors have reported the partial purification and characterization of hypothalamic somatoliberin (growth-hormone-releasing factor) from bovine, sheep and pig tissue (Dhariwal *et al.*, 1965; Schally *et al.*, 1969; Wilber *et al.*, 1971; Malacara *et al.*, 1972; Stachura *et al.*, 1972; Wilson *et al.*, 1974; Johansson *et al.*, 1974; Boyd *et al.*, 1978; Nair *et al.*, 1978). In all of these reports the active factor involved is of low molecular weight, less than 2000 (by gel filtration), in keeping with the size of the other identified hypothalamic releasing factors. The material isolated by Nair *et al.* (1978) from 6000 bovine hypothalami was active *in vivo* at doses of 100 ng, when injected into the systemic circulation, and of 10 ng when injected into the portal vessels. Enzymic studies revealed the low-molecular-weight peptide to have a pyroglutamate *N*-terminal residue, and a free *C*-terminal. The other characterized releasing factors thyroliberin (thyrotropin-releasing factor) and luliberin (luteinizing-hormone-releasing factor) also have pyroglutamate residues at their *N*-terminals. However, in contrast, somatoliberins of much larger molecular weight have been detected in extracts of tumour tissue (Beck *et al.*, 1973; Frohman *et al.*, 1980). The peptide partially purified and characterized by the latter team, from non-pituitary tumours of patients with

acromegaly, was active, *in vitro*, at doses of 40 ng of protein/ml. By gel filtration this active material had an apparent molecular weight of 8000, although a further minor peak of activity having a molecular weight in the range 15 000–30 000 was also detected. This peptide was found to have blocked *N*- and *C*-terminals.

We have previously presented chromatographic data to suggest that rat and pig hypothalamic somatoliberins have molecular weights of approx. 6000 (Sykes & Lowry, 1980), with an elution position on Sephadex G-50 between those of neurophysin and corticotropin. In the present paper we describe the purification and characterization of this somatoliberin from extracts of pig stalk median eminence.

Materials and methods

Materials

Two hundred pig stalk median eminences were obtained fresh from the Dunmow Flitch Bacon Co. Abattoir, Great Dunmow, Suffolk, U.K., within 20–40 min of slaughter, frozen on solid CO₂ and stored at –20°C until required. All extraction procedures and centrifugation steps (10 000 *g* for 40 min) were conducted at 4°C.

Sephadex G-15 (superfine grade) and Sephadex G-50 (superfine grade) were purchased from Phar-

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

macia (Uppsala, Sweden), and Bio-Gel P2 (200–400 mesh) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). A Pye–Unicam liquid-chromatography system, consisting of a pump (LC-XP pump), gradient programmer (LC-XP gradient programmer) and u.v. detector (LC3-UV detector), was purchased from Pye–Unicam (Cambridge, U.K.). H.p.l.c. columns were obtained from Altex Scientific (Berkeley, CA, U.S.A.) (Ultrasphere octadecylsilica and cyanopropylsilica), Jones Chromatography (Llanbradach, Glamorgan, Wales, U.K.), Phase Separations (Queensferry, Clwyd, Wales, U.K.) (Spherisorb octadecylsilica) and Whatman (Maidstone, Kent, U.K.) (Partisil octadecylsilica). H.p.l.c.-grade solvents and trifluoroacetic acid were purchased from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.). Amino acid standards for amino acid analysis were purchased from Calbiochem (Bishops Cleeve, Herts., U.K.), and *o*-phthalaldehyde was from Sigma Chemical Co. (Poole, Dorset, U.K.). Unless otherwise stated, all other reagents and chemicals were of analytical grade and purchased from BDH Chemicals (Poole, Dorset, U.K.).

Perfusion medium (Earle's Balanced Salts Solution) for the somatoliberin bioassay was obtained from GIBCO Biocult (Paisley, Scotland, U.K.). Human serum albumin was a gift from the Lister Institute for Preventive Medicine (London, U.K.). Trasylol was purchased from Bayer Pharmaceuticals (Basle, Switzerland), and the antibiotics benzylpenicillin and streptomycin were purchased as a combined preparation, crystamycin, from Glaxo Laboratories (Greenford, Middx., U.K.).

The reagents for the radioimmunoassay of rat somatotropin (growth hormone) and rat lutropin (luteinizing hormone) were kindly supplied by the National Pituitary Hormone Agency (Bethesda, MD, U.S.A.). The reference preparation of rat somatotropin used was rat GH-RP-4, and the antiserum was rat GH-S-4; rat somatotropin for radio-iodination was purified in our laboratory, and was fully cross-reactive with the antiserum supplied. The reference preparation of rat lutropin was rat LH-RP-1, and the antiserum was rat LH-S-3; as material for radio-iodination, rat LH-I-3 was used. Synthetic [8-arginine]vasopressin was purchased from Ferring (Malmö, Sweden), and synthetic [8-lysine]vasopressin, synthetic somatostatin and oxytocin were all purchased from Peninsula Laboratories (San Carlos, CA, U.S.A.). Native human corticotropin was purified in this laboratory (Scott & Lowry, 1974). The reagents for the radioimmunoassay of pig neurophysin were kindly supplied by Dr. Ian Robinson (National Institute for Medical Research, London, U.K.). The antisera for the oxytocin and vasopressin radioimmunoassays were a gift from Professor Tj. B. van Wimersma

Greidanus (Rudolf Magnus Institute for Pharmacology, Utrecht, The Netherlands). The vasopressin antiserum was fully cross-reactive with both [8-arginine]vasopressin and [8-lysine]vasopressin. The corticotropin-(1–39)-peptide E₄ antiserum was a gift from Wellcome Research Laboratories (Beckenham, Kent, U.K.), and the somatostatin antiserum was a gift from Dr. E. Penman (St. Bartholomew's Hospital, London, U.K.). All peptides and proteins were iodinated by using the iodogen technique (Salacinski *et al.*, 1981).

Extraction and gel filtration

The fragments of pig stalk median eminence (6.6 g) were extracted by homogenization in 60 ml of ice-cold 0.01 M-HCl in a Silverson homogenizer, and the high-molecular-weight proteins were precipitated by the addition of NaCl (5 mg/ml). After centrifugation the pellet was re-extracted with a further 20 ml of 0.01 M-HCl, NaCl (5 mg/ml) was added and the mixture was centrifuged. The combined supernatants were filtered through pre-washed glass-wool to remove lipid-like particulate matter, and the filtered extract was immediately loaded on to a Sephadex G-15 column (5 cm internal diam. × 29 cm length) connected in series to a Sephadex G-50 column (5 cm × 83 cm). The columns were equilibrated with and developed with 0.01 M-HCl/0.9% NaCl/0.1% ascorbic acid at a flow rate of 13.8 ml/h; 40 min fractions were collected at 4°C; samples were taken from every third fraction and diluted directly in the perfusion buffer.

Rat hypothalamic extract

The hypothalamic pituitary stalks (1–2 mg wet wt.) from freshly killed female Wistar rats were extracted in 0.01 M-HCl at a concentration of one fragment/ml. After the addition of NaCl (9 mg/ml) the extract was centrifuged at 10 000 g for 30 min. The supernatant was neutralized for the bioassay by the addition of 10 µl of 1.1 M-NaHCO₃/ml, or frozen at acid pH until required.

H.p.l.c.

The somatoliberin regions obtained after gel filtration of the extract of pig stalk median eminences were pooled, made 1% (v/v) with respect to trifluoroacetic acid and pumped directly on to the preparative h.p.l.c. columns. All h.p.l.c. was performed at room temperature, and the elution of peptides was monitored by recording the absorbance at 280 nm. Further details of individual h.p.l.c. runs are given in the legends to Figs. 2–5.

Amino acid analysis

Samples were hydrolysed in 6 M-HCl (with a crystal of phenol) for 24 h *in vacuo* at 115°C. Analysis was performed with a JEOL JLC-6AH automatic amino acid analyser. For micro amino

acid analysis the *o*-phthaldialdehyde fluorescence detection system of Roth (1971) was used. Cysteine residues were detected as cysteic acid after oxidation of cysteine residues with performic acid before hydrolysis.

Radioimmunoassays

Rat somatotropin was measured by using a modification of the method suggested by the N.I.A.M.D.D. Pituitary Hormone Program (J. E. C. Sykes & P. J. Lowry, unpublished work). The methodology of the radioimmunoassay of rat lutropin was the same as suggested by the Pituitary Hormone Program. The details for the radioimmunoassay for [8-arginine]vasopressin have been published previously (Gillies & Lowry, 1978). The methodology for the oxytocin immunoassay was identical with that of the vasopressin immunoassay, and that for neurophysin was identical with the somatotropin immunoassay. The corticotropin radioimmunoassay method was that of Rees *et al.* (1971), and the somatostatin immunoassay method followed that of Penman *et al.* (1979).

Somatoliberin bioassay

The perfused isolated rat anterior-pituitary-cell column of Gillies & Lowry (1978) was adapted as a bioassay for somatoliberin as described previously (Sykes & Lowry, 1980; Lowry *et al.*, 1980). Briefly, the anterior-pituitary cells from female Wistar rats (200–250 g) were dispersed by trypsinization

(0.25%) and mechanical agitation, and mixed with 0.5 g Bio-Gel P2 pre-swollen in 0.9% NaCl, and washed with perfusion medium containing antibiotics (25 µg of streptomycin/ml and 15 µg of benzylpenicillin/ml) and ascorbic acid (30 µg/ml). The suspension of cells and Bio-Gel was packed into a small plastic column and perfused with perfusion medium containing human serum albumin (0.25%) and Trasylol (100 kallikrein-inhibitory units/ml) in addition to the antibiotics and ascorbic acid, at a flow rate of 30 ml/h. After a 1.5 h equilibration period the cell column was stimulated with test material for 2 min every 10 min; 2 min fractions were collected and assayed for rat somatotropin by radioimmunoassay.

Treatment of data

Rat somatotropin was expressed in terms of the N.I.A.M.D.D. reference preparation, and was the total amount of somatotropin released above background. To establish a logarithmic relationship with the dose, samples were applied randomly in quadruplicate and the results were analysed by using Student's *t* test and regression analysis.

Results

Gel filtration

Samples were tested for somatoliberin activity by bioassay at a dilution of 1:200. As shown in Fig. 1,

Fig. 1. Gel filtration of an extract of pig stalk median eminence

A fractionated extract (75 ml) of pig stalk median eminence was gel-filtered on a Sephadex G-15 column (5 cm internal diam. × 29 cm length) and a Sephadex G-50 column (5 cm × 83 cm) connected in series; the eluent buffer was 0.01 M-HCl/0.9% NaCl/0.1% ascorbic acid. The flow rate was 13.8 ml/h; 40 min fractions (9.9 ml) were collected at 4°C, and every third fraction was assayed for somatoliberin bioactivity (▲) and somatostatin immunoreactivity (■). The void volume of the column was calculated at 838 ml; the first 690 ml of the effluent was discarded before the collection of timed fractions. Key to markers; NP, neurophysin; ACTH, corticotropin; OT, oxytocin; LVP, [8-lysine]vasopressin.

two somatoliberin active regions were detected. The first, more potent, region of activity was eluted between immunoreactive pig neurophysin and corticotropin with $K_{av} = 0.4$, in a similar position to the somatoliberin activity observed previously in the chromatograms of rat stalk median eminence (Sykes & Lowry, 1980). The later double peak of somatoliberin activity ($K_{av} = 0.84$) was eluted in the region of immunoreactive oxytocin and vasopressin. Multiple molecular-weight forms of somatostatin were observed, with approximate molecular weights of $>20\,000$ ($K_{av} = 0$), $12\,000$ ($K_{av} = 0.34$), $4\,000$ ($K_{av} = 0.56$) and $1\,000$ – $2\,000$ ($K_{av} = 0.79$ – 1.12). The major peak of immunoreactive somatostatin was that with a K_{av} of 0.56 . Other authors have reported high-molecular-weight forms of somatostatin with approximate molecular weights of $25\,000$ (Zingg & Patel, 1979), $12\,000$, $4\,000$ and $2\,000$ (Conlon *et al.*, 1978; Noe *et al.*, 1978; Schally *et al.*, 1980; Spiess & Vale, 1980).

Both the somatoliberin-active regions were further purified by h.p.l.c.

Preparative and analytical h.p.l.c. of the minor somatoliberin-active region

Fractions from the oxytocin/vasopressin-associated somatoliberin-active region (fractions

121–141 in Fig. 1) were pooled and submitted to preparative h.p.l.c. on a column of Spherisorb octadecylsilica ($10\,\mu\text{m}$). Samples from fractions 1–32 inclusive were tested for somatoliberin activity in the bioassay at a dilution of 1:100, and for vasopressin and oxytocin by radioimmunoassay. As shown in Fig. 2, release of somatotropin was associated with the regions of immunoreactive vasopressin and oxytocin (retention times 3.3 and 6.9 min respectively). Further purification of the peak immunoreactive tubes, fractions 11 and 23, by analytical h.p.l.c. [Partisil octadecylsilica ($0.4\,\text{cm} \times 25\,\text{cm}$); 0–80% (v/v) methanol in 1% (v/v) trifluoroacetic acid] followed by amino acid analysis, purified fraction 11 giving Asx (0.88), Glx (1.19), Pro (1.19), Gly (1.25), Cys (1.7), Leu (2.45), Tyr (0.88), Phe (1.0) and Lys (1.13), and purified fraction 23 giving Asx (1.04), Glx (1.04), Pro (1.26), Gly (1.43), Cys (2.3), Ile (1.15), Leu (1.15) and Tyr (1.04), confirmed the substances to be [8-lysine]vasopressin and oxytocin.

Preparative and analytical h.p.l.c. of the major somatoliberin-active region

Two strongly absorbing peaks were observed (Fig. 3), which were eluted at 59% and 62% of solvent B (retention times of 2.4 and 5.6 min

Fig. 2. Preparative h.p.l.c. of the 'minor' somatoliberin bioactive region of gel-filtered extract of pig stalk median eminence

The 'minor' somatoliberin-bioactive region was obtained after gel filtration of a fractionated extract of pig stalk median eminence. Alternate fractions (121–141, Fig. 1) were pooled and loaded on to a column ($0.5\,\text{cm} \times 10\,\text{cm}$) of Spherisorb octadecylsilica ($10\,\mu\text{m}$). A 50 min linear gradient of 0% (solvent A) to 80% (solvent B) (v/v) methanol in 1% (v/v) trifluoroacetic acid was applied at a flow rate of $2\,\text{ml/min}$; 0.3 min fractions were collected and assayed for somatoliberin bioactivity (●) and for [8-lysine]vasopressin (□) and oxytocin (Δ) immunoreactivity. The upper diagram shows the absorbance of peptides at $280\,\text{nm}$ (○—○) eluted by the solvent gradient (—). The lower diagram indicates the elution positions of bioactive somatoliberin and immunoreactive vasopressin and oxytocin.

Fig. 3. *Preparative h.p.l.c. of pig hypothalamic (stalk-median-eminece) somatoliberin*

The somatoliberin-bioactive region obtained after gel filtration of a fractionated extract of pig stalk median eminence (alternate fractions 63–77, Fig. 1) was pooled and applied to a column (1 cm × 15 cm) of Ultrasphere octadecylsilica (5 µm). Peptides were eluted by a 30 min linear gradient of 0% (solvent A) to 80% (solvent B) (v/v) methanol in 1% (v/v) trifluoroacetic acid. The flow rate was 3.5 ml/min; 0.2 min fractions were collected and assayed for somatoliberin bioactivity (▲) and for immunoreactive neurophysin (■). The absorbance of peptides at 280 nm (—) and the solvent gradient (----) are also shown. The somatoliberin fractions (109–111) were further purified by analytical h.p.l.c.

respectively) and which were well separated from the other u.v.-absorbing material. These peaks did not cross-react in either the vasopressin or the oxytocin radioimmunoassays. Samples from the peak tubes (fractions 12 and 28) were taken for amino acid analysis, with the use of both ninhydrin and o-phthalaldehyde fluorescence detection of amino acids. As shown in Table 1, the two peptides were not of similar composition and did not correspond to any previously described peptide.

The somatoliberin-bioactive material (tested at a dilution of 1:200) was eluted as a broad peak in 90–92% solvent B (retention time 21.8–22.2 min) between two incompletely separated peaks of immunoreactive neurophysin. Neither the somatoliberin bioactivity nor the neurophysin immunoreactivity was eluted as a well-defined u.v.-absorbing peak, but occurred in a broad region of u.v. absorbance. Peak tubes of immunoreactive neurophysin contained 25.2 µg and 35.2 µg of neurophysin respectively. After gel filtration the peak tube of immunoreactive neurophysin contained 93.6 µg of neurophysin, indicating a loss of 39% of immunoreactive neurophysin during preparative h.p.l.c.

The main somatoliberin-containing fractions (109–111 in Fig. 3) were further purified by h.p.l.c. on a cyanopropylsilica column. Samples were tested for somatoliberin activity (at a 1:200 dilution) and for immunoreactive neurophysin. There was a near-complete separation of somatoliberin bioactivity from neurophysin immunoreactivity (Fig. 4). Somatoliberin was eluted in 75% solvent B (retention time 7.2 min), whereas immunoreactive neurophysin was associated with the smaller, later-eluted, peak occurring at 85% solvent B (retention time 9.0 min). By radioimmunoassay, the recovery of immunoreactive neurophysin was 99%.

Table 1. *Amino acid compositions of the early eluted (59% and 62% solvent B) peaks from the preparative h.p.l.c. of pig somatoliberin (Fig 1) and amino acid composition of purified pig somatoliberin (fraction 46/47, Fig. 5)*

For experimental details see the text. The compositions are based on the assumption of 1 histidine residue/molecule in each case.

Amino acid	Amino acid composition (mol of residue/mol)		Somatoliberin
	59%-solvent B peak	62%-solvent B peak	
Asx	2.6	2.8	4.9
Thr*	0.7	0.4	1.3
Ser*	2.8	1.9	5.4
Glx	9.0	4.2	11.2
Pro	1.5	1.2	3.3
Gly	2.9	3.5	4.0
Ala	5.1	4.1	6.3
Cys	1.1	2.2	—
Val	1.3	1.5	2.7
Met	0.9	0.5	0.6
Ile	1.0	1.2	2.4
Leu	1.4	2.8	4.2
Tyr	4.4	1.0	1.4
Phe	0.9	0.6	1.8
His	1.0	1.0	1.0
Lys	3.2	3.0	3.7
Arg	3.9	2.8	2.8
Trp	2.7	2.1	0–1

* Corrected for 20% loss during hydrolysis.

Fractions 23 and 24 (Fig. 4) were applied to a further analytical h.p.l.c. column. The somatoliberin-containing shoulder was resolved into many components. Somatoliberin activity (tested at a

Fig. 4. Analytical h.p.l.c. of pig hypothalamic (stalk-median-eminence) somatoliberin

The somatoliberin-bioactive fractions (109–111, Fig. 3) obtained by preparative h.p.l.c. were pooled and injected on to a column (0.5 cm $\times$ 15 cm) of Ultrasphere cyanopropylsilica (5 μ m). Peptides were eluted by a 30 min linear gradient of 0% (solvent A) to 80% (solvent B) (v/v) methanol in 1% (v/v) trifluoroacetic acid. The flow rate was 1 ml/min; 0.3 min fractions were collected and assayed for somatoliberin bioactivity ($\blacktriangle$) and immunoreactive neurophysin ($\blacksquare$). The absorbance of peptides at 280 nm (—) and the solvent gradient (----) are also shown. Fractions 23 and 24 were further purified by analytical h.p.l.c. on Ultrasphere octadecylsilica.

dilution of 1:500) was associated with the main u.v.-absorbing peak (retention time 9.2 min; elution in 87% solvent B). Immunoreactive neurophysin was eluted before the somatoliberin-active peak, with a retention time of 8.0 min.

Samples from fractions 46 and 47 (Fig. 5) were taken for amino acid analysis, with the use of both ninhydrin and *o*-phthalaldehyde fluorescence detection of amino acid residues. Fraction 47 was oxidized with performic acid to permit the detection of cysteine (as cysteic acid). The results are shown in Table 1. The somatoliberin material was characterized by a high number of glutamate (or glutamine) residues, with aspartate (or asparagine) being the next predominant residue. Cysteic acid was not detected in the hydrolysate of fraction 47. Amino sugars were also not detected. Tryptophan residues were estimated as 0–1/molecule, calculated from the absorbance at 280 nm. There was only 1 tyrosine

residue/molecule. Thus the pig somatoliberin molecule has about 56–57 residues, which would give a molecular weight of approx. 6400, consistent with its elution position on Sephadex G-50 between immunoreactive neurophysin and corticotropin.

The material from fraction 46 and 47 (Fig. 5) was very active in releasing somatotropin from the anterior-pituitary-cell column over the dilution range 1:8000–1:500 (approx. 0.1–10 ng of peptide), and gave a steep dose-response curve ($P < 0.005$) (Fig. 6). When matched for the dose/dilution required to give a release of 450 ng of rat somatotropin/ml, the regression coefficient, *B*, for pig somatoliberin was 3378 and for an extract of rat stalk median eminence was 2323. Neither corticotropin nor rat lutropin was released from the anterior-pituitary-cell bioassay after stimulation with this large-molecular-weight pig stalk-median-eminence somatoliberin.

Fig. 5. Final purification of pig hypothalamic (stalk-median-eminence) somatoliberin by analytical h.p.l.c.

The somatoliberin-bioactive fractions (23 and 24) obtained after h.p.l.c. on cyanopropylsilica (Fig. 4) were loaded on to a column (0.5 cm $\times$ 25 cm) of Ultrasphere octadecylsilica (5 μ m). A 30 min linear gradient of 0% (solvent A) to 60% (solvent B) (v/v) acetonitrile in 1% (v/v) trifluoroacetic acid was applied at a flow rate of 1 ml/min; 0.2 min fractions were collected and assayed for somatoliberin bioactivity ($\blacktriangle$) and immunoreactive neurophysin ($\blacksquare$). The absorbance of the peptides at 280 nm (—) and the solvent gradient (----) are also shown. Samples of the fractions 46 and 47 were subjected to acid hydrolysis for amino acid analysis.

Discussion

Pig hypothalamic somatoliberin activity was purified from an acid extract of pig stalk median eminence by gel filtration and preparative and analytical h.p.l.c. The final purification step, yielding less than 16 μ g of active material, resulted in a near-complete separation of somatoliberin from its main contaminant (neurophysin). Final amino acid analysis both before and after performic acid oxidation, with the use of both ninhydrin and *o*-phthalaldehyde fluorescence detection, revealed a large peptide with a high number of glutamate (or glutamine) and aspartate (or asparagine) residues (accounting for 28% of the total residues), no cysteine and low amounts of methionine, threonine, tyrosine, phenylalanine and histidine. Basic amino acid residues (arginine and lysine) accounted for 12% of the total peptide. Tryptophan was estimated

at 0–1 residues/molecule. The lack of cysteine in the final amino acid analysis indicated that the somatoliberin fraction analysed was free from neurophysin.

With about 56–57 amino acids, the peptide molecule would have a molecular weight of approx. 6400, in agreement with the suggested molecular weight of the tumour somatoliberin reported by Frohman *et al.* (1980). Our peptide also appears to have a great similarity to that found by Schally *et al.* (1969) in that both have a high glutamate content. However, our material is distinguished by a larger molecular weight and the presence of additional amino acids (namely arginine, proline, threonine, glycine, methionine, phenylalanine, isoleucine and aspartate). The lack of cysteine in our material is in agreement with the observation by Arimura *et al.* (1967) that thioglycollate failed to inactivate hypothalamic somatoliberin.

Fig. 6. Dose-response curves of purified pig stalk-median-eminenence somatoliberin and an extract of rat stalk median eminence

Purified pig stalk-median-eminenence somatoliberin (●) (fraction 46, Fig. 5) and a crude extract of rat stalk median eminence (■) were prepared as described in the Materials and methods section. At dose/dilution 1 both secretagogues released approx. 450 ng of rat somatotropin/ml.

The difficulty in freeing hypothalamic somatoliberin from immunoreactive neurophysin, together with our earlier observation that both oxytocin and vasopressin, both bound by the neurophysins, stimulated the release of rat somatotropin from the anterior-pituitary-cell column (Sykes & Lowry, 1980; J. E. C. Sykes & P. J. Lowry, unpublished work), raised the possibility that the peptide isolated was part of a complex, analogous to the proposed corticoliberin-releasing factor complex (consisting of [8-arginine]vasopressin acting synergistically with the other factors; Gillies & Lowry, 1979, 1980). Furthermore, the somatoliberin- and oxytocin-stimulated release of rat somatotropin was inhibited by synthetic somatostatin in a dose-dependent manner, and with parallel dose-inhibition curves (J. E. C. Sykes & P. J. Lowry, unpublished work). However, the large-molecular-weight pig somatoliberin released only somatotropin, and thus must be considered as the major physiological somatoliberin, whereas both oxytocin and vasopressin are capable of releasing other adenohypophysial hormones (Gillies & Lowry, 1978; J. E. C. Sykes & P. J. Lowry, unpublished work).

A somatoliberin with a molecular weight greater than that of the previously identified hypothalamic factors is implied by the properties of the somatoliberin receptor proposed by Momany *et al.* (1981). Furthermore, Guillemin (1980) described the isolation from hypophysial and neurohypophysial

extracts of a somatotropin-releasing peptide that had 35–37 amino acid residues/molecule, including 2 cysteine residues. More recently, Böhlen *et al.* (1982) have reported the isolation of a tumour somatoliberin with an estimated molecular weight of about 5000.

It is difficult to equate the undoubted somatotropin-releasing activity of the small peptide isolated by Nair *et al.* (1978) with the specific release of rat somatotropin *in vitro* caused by our much larger peptide. In view of the stimulatory effect *in vivo* of the former group's peptide on somatotropin synthesis and release, it is difficult to envisage their material as being an artifact. Like Frohman *et al.* (1980), we must conclude (a) that the two peptides are unrelated or (b) that our material represents a precursor somatoliberin, 'big-somatoliberin', with full or enhanced bioactivity. In view of the reported greater bioactivity of somatostatin-28 compared with somatostatin-14 in inhibiting somatotropin release (Spiess & Vale, 1980), there always remains the possibility that the smaller hypothalamic peptides isolated so far are only bioactive fragments of a larger molecule. This could also be implied from the observation by Vale *et al.* (1981) that only the C-terminal region of the 41-residue sheep corticoliberin (corticotropin-releasing factor) was required for full activity, allowing the possibility that smaller corticoliberin-like peptides could exist that are N-terminally shortened but that still retain the bioactive C-terminal. Interestingly, trypsin treatment of a partially purified preparation of pig somatoliberin only partially abolished its somatotropin-releasing properties (J. E. C. Sykes & P. J. Lowry, unpublished work). Thus the large-molecular-weight somatoliberin described in the present paper could represent the precursor of the smaller peptides described by others.

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References

- Arimura, A., Sawano, S., Bowers, C. Y. & Schally, A. V. (1967) *Endocrinology* **81**, 1165–1168
- Beck, C., Larkins, R. G., Martin, J. J. & Burger, H. G. (1973) *Endocrinology* **59**, 325–333
- Böhlen, P., Thorner, M., Cronin, M., Shively, J. & Scheithauer, B. (1982) *Annu. Meet. Am. Endocrine Soc. 64th*, abstr. 540
- Boyd, A. E., Sanchez-Franco, F., Spencer, E., Patel, Y. C., Jackson, I. M. D. & Reichlin, S. (1978) *Endocrinology* **103**, 1075–1080
- Conlon, J. M., Zyznar, E., Vale, W. & Unger, R. H. (1978) *FEBS Lett.* **94**, 327–330
- Dhariwal, A. P. S., Krulich, L., Katz, S. H. & McCann, S. M. (1965) *Endocrinology* **77**, 932–936
- Frohman, L. A., Szabo, M., Berelowitz, M. & Stachura, M. (1980) *J. Clin. Invest.* **65**, 43–54

- Gillies, G. E. & Lowry, P. J. (1978) *Endocrinology* **103**, 521–527
- Gillies, G. E. & Lowry, P. J. (1979) *Nature (London)* **278**, 463–464
- Gillies, G. E. & Lowry, P. J. (1980) *J. Endocrinol.* **86**, 205–219
- Guillemin, R. (1980) *J. Endocrinol.* **90**, 3P–10P
- Johansson, K. N.-G., Currie, B., Folkers, K. & Bowers, C. Y. (1974) *Biochem. Biophys. Res. Commun.* **60**, 610–615
- Lowry, P. J., Sykes, J. E. C. & Gillies, G. E. (1980) *Adv. Biochem. Pharmacol.* **22**, 119–129
- Malacara, J. M., Valverde-R, C., Reichlin, S. & Bollinger, J. (1972) *Endocrinology* **91**, 1189–1198
- Momany, F. A., Bowers, C. Y., Reynolds, G. A., Chang, G., Hong, A. & Newlander, K. (1981) *Endocrinology* **108**, 31–39
- Nair, R. M. G., de Villier, C., Barnes, M., Anatalis, J. & Wilbur, D. L. (1978) *Endocrinology* **103**, 112–120
- Noe, B. D., Weir, G. C. & Bauer, G. E. (1978) *Metab. Clin. Exp.* **27**, 1201–1205
- Penman, E., Wass, J. A. H., Lund, A., Lowry, P. J., Stewart, J., Dawson, A. M., Besser, G. M. & Rees, L. H. (1979) *Ann. Clin. Biochem.* **16**, 15–25
- Rees, L. H., Cook, D. M., Kendall, J. W., Allen, O. F., Kramer, R. M., Ratcliffe, J. G. & Knight, R. A. (1971) *Endocrinology* **89**, 254–261
- Roth, M. (1971) *Anal. Chem.* **43**, 880–882
- Salacinski, P. R., McLean, C., Clement-Jones, V., Sykes, J. E. C. & Lowry, P. J. (1981) *Anal. Biochem.* **117**, 136–146
- Schally, A. V., Sawano, S., Arimura, A., Barrett, J. F., Wakabayashi, I & Bowers, C. Y. (1969) *Endocrinology* **84**, 1493–1506
- Schally, A. V., Huang, W.-Y., Chang, R. C. C., Arimura, A., Reeding, T. W., Millar, R. P., Hunkapillar, M. W. & Hood, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4489–4493
- Scott, A. P. & Lowry, P. J. (1974) *Biochem. J.* **139**, 593–602
- Spiess, J. & Vale, W. (1980) *Biochemistry* **19**, 2861–2866
- Stachura, M. E., Dhariwal, A. P. S. & Frohman, L. A. (1972) *Endocrinology* **91**, 1071–1078
- Sykes, J. E. C. & Lowry, P. J. (1980) *J. Endocrinol.* **85**, 42P–43P
- Vale, W., Spiess, J., Rivier, C. & Rivier, J. (1981) *Science* **213**, 1394–1397
- Wilber, J. F., Nagel, T. & White, W. F. (1971) *Endocrinology* **89**, 1419–1424
- Wilson, M. C., Steiner, A. L., Dhariwal, A. P. S. & Peake, G. T. (1974) *Neuroendocrinology* **15**, 313–327
- Zingg, H. H. & Patel, Y. C. (1979) *Biochem. Biophys. Res. Commun.* **90**, 466–472