Characterization of immunoreactive somatostatin released from rat nervous system *in vitro*

Michael C. SHEPPARD,* Salie HENDRICKS,† Anne HUDSON† and Shirley R. KRONHEIM†

*Department of Medicine, University of Birmingham, Queen Elizabeth Hospital, Birmingham, U.K., and

†Department of Medicine, University of Cape Town, Cape Town, Republic of South Africa

(Received 22 July 1981/Accepted 12 October 1981)

There is considerable evidence that somatostatin is released from nerve terminals throughout the central nervous system in response to presynaptic stimulation, thus suggesting a neuromodulator role for the peptide. We here report the partial characterization of immunoreactive somatostatin released from rat nervous system *in vitro* (hypothalamus, spinal cord and hypothalamic, cortical, thalamic and striatal synaptosomes). Serial dilutions of released somatostatin immunoreactivity showed parallelism with dilutions of synthetic somatostatin standard. Somatostatin immunoreactivity released from all tissue areas coeluted with synthetic tetradecapeptide on Sephadex G-25 (fine-grade) gel chromatography; more than 85% of this immunoreactivity bound to Sepharose–anti-somatostatin-serum immunoaffinity columns. In addition, immunoreactive material released from hypothalamus, spinal cord and hypothalamic and cortical synaptosomes inhibited somatotropin (growth hormone, ‘STH’, ‘GH’) release from perfused anterior pituitary in a dose-related manner, indicating biological similarity to synthetic somatostatin.

Somatostatin has a widespread and uneven distribution throughout mammalian brain, possesses direct depressant effects on central-neuronal function as well as a number of behavioural effects, and is localized in nerve terminals, synaptosomes and secretory granules (Krieger & Martin, 1981).

The release of immunoreactive somatostatin has been described from a variety of rat central-nervous-system preparations *in vitro*, including incubated hypothalami (Berelowitz *et al.*, 1978a), perfused mediobasal hypothalami (Terry *et al.*, 1980), incubated median eminence fragments (Negro-Vilar *et al.*, 1978), neurohypophysis (Patel *et al.*, 1977), superfused slices of rat hypothalamus and amygdala (Iversen *et al.*, 1978), synaptosomes isolated from various areas of rat brain (Bennett *et al.*, 1979), and, in addition, rat spinal cord (Sheppard *et al.*, 1979). In all instances the release of somatostatin shares two important characteristics with the neuro-secretory process: stimulation of release in response to membrane depolarization and dependence of this process on extracellular Ca^{2+} . There is thus strong evidence that somatostatin may have a neuromodulator role in the nervous system, being released from nerve terminals throughout the brain in

response to presynaptic stimulation, in addition to its probable hypophysiotrophic function in regulating somatotropin (growth hormone, ‘STH’, ‘GH’) secretion. Although radioimmunoassays used for the measurement of somatostatin under these conditions are specific, in that no cross-reactivity has been observed with a large number of peptides and hormones, true identity of the measured immunoreactivity with synthetic tetradecapeptide somatostatin still remains to be proven. In the present study we report the partial characterization of immunoreactive somatostatin released from incubated rat hypothalamus (Berelowitz *et al.*, 1978a), synaptosomes isolated from rat brain (Bennett *et al.*, 1979; Sheppard, 1981), and incubated rat spinal cord (Sheppard *et al.*, 1979) by means of: (1) comparison of serial dilutions of sample immunoreactivity with the radioimmunoassay dose-response curve; (2) Sephadex G-25 (fine-grade) gel chromatography; (3) immunoaffinity chromatography; and (4) inhibition of somatotropin secretion from the rat pituitary *in vitro*. Although it is accepted that final identification of the measured immunoreactive somatostatin depends on amino acid sequencing, the demonstration of identity of immunoreactivity with synthetic tetradecapeptide somatostatin in the four systems described would be

Abbreviation used: $^{125}\text{ITyr}$, [^{125}I]iodotyrosine.

strong evidence for the released material being biologically active tetradecapeptide somatostatin.

Materials and methods

Rat hypothalami, spinal cord and rat brain synaptosomes were incubated under carefully controlled conditions as previously described in detail (Berelowitz *et al.*, 1978a; Sheppard *et al.*, 1979; Bennett *et al.*, 1979; Berelowitz *et al.*, 1978b). Medium was frozen and stored at -20°C after the incubation period. Somatostatin was measured by radioimmunoassay as previously detailed (Kronheim *et al.*, 1976).

Comparison of serial dilutions of sample immunoreactivity with the radioimmunoassay dose-response curve

Measured immunoreactivity in an unknown sample and synthetic standard must be shown to react identically with the antibody (Yalow & Berson, 1971). This can be done by demonstrating that inhibition curves generated in the immunoassay by somatostatin immunoreactivity are parallel to the inhibition curve produced by synthetic standard. Serial dilutions of medium immunoreactivity obtained from all tissue preparations were prepared in the immunoassay buffer and the inhibition curves generated in the immunoassay compared with those obtained by dilution of somatostatin standards. The results were analysed by linear transformation of the inhibition curves to enable comparison of slopes (Feldman & Rodbard, 1971). $[^{125}\text{ITyr}^1]\text{somatostatin}$ binding to antibody (B) was expressed as $\log B/B_0$ where $B_0 = [^{125}\text{ITyr}^1]\text{somatostatin}$ binding to antibody in the absence of unlabelled somatostatin. $\log B/B_0$ was plotted on the ordinate against $\log$ dose or dilution on the abscissa and the data subjected to linear-regression analysis, allowing the regression coefficient (b) and correlation coefficient (r) of each line to be calculated.

Gel chromatography

Sephadex gels have been used extensively in the purification and characterization of somatostatin (Coy *et al.*, 1973; Yamashiro & Li, 1973; Rivier, 1974). For the purposes of the present study, Sephadex G-25 (fine grade) was used throughout. The gel was swollen in 1 M-acetic acid at room temperature for 3 h and degassed before pouring 1.6 cm $\times$ 90 cm or 1.6 cm $\times$ 94 cm columns, which were kept at 4°C . A constant flow rate of 30 ml/h was maintained by means of a peristaltic pump (LKB). Samples (2 ml) of medium removed from hypothalamus and spinal cord after 30 min of incubation, and of supernatant from synaptosome suspension centrifuged after 30 min incubation, were applied to the column. Fractions (5 ml) were eluted

with 1 M-acetic acid, freeze-dried and reconstituted in immunoassay buffer before being analysed for somatostatin immunoreactivity. The column was calibrated with Dextran Blue (mol.wt. 2000000) to define the void volume, synthetic tetradecapeptide somatostatin (Ayerst AY24910, mol.wt. 1639) and urea (mol.wt. 79). Results were expressed in terms of the retention constant, V_0/V_e , where V_0 is the void volume and V_e is the elution volume of the sample.

Affinity chromatography

To determine whether measured somatostatin immunoreactivity was truly immunoreactive and not the result of non-specific immunoassay interference, CNBr-activated Sepharose was coupled to precipitates of rabbit anti-somatostatin serum and non-immune rabbit serum. Columns were prepared of both, and the elution patterns of sample and synthetic standard compared. A 40%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate of somatostatin antiserum was dialysed and coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), the conjugate was washed and the binding sites that had not reacted were blocked with 1 M-ethanolamine. Columns (1 cm $\times$ 1 cm) were prepared, equilibrated with 0.01 M-phosphate in 0.015 M-NaCl, pH 7.8 (phosphate-buffered saline), and the sample (synthetic tetradecapeptide somatostatin or released immunoreactive material from hypothalamus, spinal cord and hypothalamic and cortical synaptosomes) was recycled for 2 h by using peristaltic pumps (LKB) at a flow rate of 30 ml/h. After this time, ten 2 ml fractions were eluted with phosphate-buffered saline to recover non-antibody-bound assayable material; a further ten 2 ml fractions were eluted with 1 M-acetic acid to remove the bound material. Controls consisted of columns containing Sepharose conjugated to non-immune rabbit serum. Non-specific adsorption to the column was calculated from the amount of material applied and the total eluted with both phosphate-buffered saline and acetic acid. Standard or sample immunoreactivity eluted with phosphate-buffered saline and with acetic acid was expressed as a percentage of total material eluted from the column.

Biological activity of released immunoreactive somatostatin

To determine whether the immunoreactive somatostatin released from rat brain preparations *in vitro* possessed biological activity, the material was tested in a rat hemipituitary perfusion system. To ensure that biological activity of only immunoreactive somatostatin was being assessed, acetic acid eluates from the above-described Sepharose-somatostatin antiserum affinity columns were freeze-dried, resuspended in perfusion medium and tested in the hemipituitary system.

Anterior pituitary glands were obtained from male Long-Evans rats weighing 200–250g. Rats were decapitated, their brains reflected and removed, and the pituitary rapidly dissected free from the fossa. The posterior pituitary was excised and discarded and the anterior pituitary halved. A chamber to hold the tissue was devised from a 2 ml disposable syringe with inflow via a needle inserted through the rubber piston; the volume of contents was decreased to 0.2 ml during experiments. Two hemipituitaries (from different animals) were placed in the reaction chamber, which was then submerged in a water bath at 37°C. Modified Gey and Gey buffer (see below), equilibrated with O₂/CO₂ (19:1), was pumped through two such chambers (each containing two hemipituitaries) by means of an LKB multiperplex pump for 2½ h of preincubation and stabilization. The effluent (perfusate) thereafter was collected in 2 ml portions (equivalent to 4 min) on a fraction collector (LKB). Modified Gey and Gey buffer (Carlson *et al.*, 1974) comprised: NaCl, 111 mM; KCl, 3.7 mM; KH₂PO₄, 0.22 mM; NaHCO₃, 27 mM; MgSO₄, 0.28 mM; MgCl₂, 1.03 mM; CaCl₂, 2.64 mM; Na₂HPO₄, 0.79 mM; D-glucose, 0.1% (w/v); and bovine serum albumin, 1% (w/v; Miles, fraction V).

Test substances were dissolved in the above medium to give final concentrations in the reaction chamber of: (1) theophylline (Calbiochem), 0.5, 1.5, 2.5, 5.5 and 10.0 mM; and (2) synthetic tetradecapeptide somatostatin (Ayerst 24910), 0.05, 0.10, 0.25, 0.50 and 1.00 ng/ml. In addition, 1 M-acetic acid eluates from coupled Sepharose–somatostatin antiserum columns containing immunoreactive somatostatin released from hypothalamus, hypothalamic and cortical synaptosomes, and spinal cord were freeze-dried, resuspended in perfusion buffer and diluted serially in buffer. These test substances were introduced into the system via a three-way metal valve and each perfusate fraction was assayed for rat somatotropin with reagents supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD).

Viability of the hemipituitary preparation was assessed by the Warburg technique in medium comprising: NaCl, 119 mM; KCl, 4.7 mM; KH₂PO₄, 1.2 mM; Na₂HPO₄, 20 mM; MgSO₄, 1.2 mM; and CaCl₂, 2.5 mM, equilibrated with 100% O₂.

The following perfusion experiments were performed: (1) dose–response effect of theophylline on basal rat somatotropin release; (2) dose–response effect of synthetic somatostatin on 2.5 mM-theophylline-stimulated rat somatotropin release; and (3) effect of serial dilutions of immunoreactive somatostatin on 2.5 mM-theophylline-stimulated rat somatotropin release. The calculation of results was performed as follows.

(1) Basal somatotropin release (B) = mean value for four fractions before stimulus (after 150 min

preincubation period). Somatotropin response to stimulus (R) = mean value for four fractions after stimulus.

(2) For accuracy and reproducibility each response (of test substance) was defined in terms of the preceding basal values and expressed as a percentage:

$$\frac{\text{Response (R)}}{\text{Basal (B)}} \times 100$$

(3) For experiments determining inhibitory effect of somatostatin or released somatostatin immunoreactivity, the following calculation was employed:

$$\text{Percentage inhibition} = 100 - \left[\frac{R_2/B_2}{R_1/B_1} \times 100 \right]$$

where R₂ = mean response to theophylline + somatostatin; B₂ = mean basal value before stimulus; R₁ = mean response to theophylline alone; B₁ = mean basal value before stimulus.

(4) For comparison of biological effect of serial dilutions of somatostatin immunoreactivity with somatostatin standard, a logit–log transformation was applied to the data to render the inhibitory responses linear and thus log (concentration of standard) or log dilution was plotted against:

$$\text{logit} \left[1 - \frac{R_2/B_2}{R_1/B_1} \right] \text{ (as defined above)}$$

Results

Comparison of sample immunoreactivity dilutions with radioimmunoassay dose-response curve

A logit–log plot of serial dilutions of medium containing immunoreactive somatostatin released from incubated hypothalamus ($b = 2.063$, $r = 0.946$) and spinal cord ($b = 2.118$, $r = 0.843$) showed parallelism with decreasing amounts of synthetic somatostatin ($b = -2.238$, $r = 0.978$; Fig. 1). Similar logit–log plots (Figs. 2 and 3) of serial dilutions of released immunoreactivity from isolated hypothalamic synaptosomes ($b = -2.178$, $r = 0.986$), cortical synaptosomes ($b = 2.294$, $r = 0.972$), thalamic synaptosomes ($b = 2.236$, $r = 0.939$) and striatal synaptosomes ($b = -2.170$, $r = 0.973$) also showed parallelism with a logit–log transformation of the standard dose–response curve ($b = -2.216$, $r = 0.986$). A slope with regression coefficient (b) within two standard deviations of the regression coefficient of the somatostatin standard slope was considered to be not significantly different. Serial dilutions of samples obtained from all preparations were thus linear and showed parallelism with dilutions of somatostatin standard when inhibition curves were subjected to logit–log transformation.

Fig. 1. Logit-log plot of serial dilutions of immunoreactive somatostatin released from suspensions of hypothalamus (□) ($n = 21$) and spinal cord (▼) ($n = 29$), showing parallelism with decreasing concentrations of synthetic somatostatin (○) ($n = 20$)

Fig. 2. Logit-log plot of serial dilutions of immunoreactive somatostatin released from suspensions of hypothalamic synatosomes (□) ($n = 14$) and thalamic synatosomes (▼) ($n = 11$), showing parallelism with decreasing concentrations of synthetic somatostatin (○) ($n = 24$)

Fig. 3. Logit-log plot of serial dilutions of immunoreactive somatostatin released from suspensions of striatal synatosomes (▼) ($n = 10$) and cortical synatosomes (□) ($n = 15$), showing parallelism with decreasing concentrations of synthetic somatostatin (○) ($n = 24$)

Gel chromatography

Somatostatin immunoreactivity released from hypothalamus, spinal cord and isolated synatosomes was eluted as a single peak in all instances and showed identity with eluted synthetic tetradecapeptide somatostatin (Figs. 4, 5 and 6). Recovery of synthetic somatostatin from the column was $40.1 \pm 2.8\%$ (mean $\pm$ S.E.M., $n = 6$). The recovery of somatostatin immunoreactivity from the column was as follows: hypothalamus, $42.6 \pm 5.2\%$; spinal cord, $39.8 \pm 4.8\%$; hypothalamic synatosomes, $53.9 \pm 5.3\%$; cortical synatosomes, $44.4 \pm 6.2\%$; thalamic synatosomes, $46.2 \pm 5.5\%$; and striatal synatosomes, $54.0 \pm 7.0\%$. Results are expressed as means $\pm$ S.E.M., $n = 3$ in all instances.

Affinity chromatography (Table 1)

Recovery of standard or sample immunoreactivity was more than 80% in all instances. The major proportion of standard or sample immunoreactivity (more than 85%) bound to the Sepharose-anti-somatostatin-serum conjugate, whereas very little of the material bound to the Sepharose-non-immune-rabbit-serum column, indicating that the immunoassayable material was truly immunoreactive.

Biological activity of released immunoreactive somatostatin

(1) Viability of the hemipituitary preparation in

Fig. 4. Elution profiles of immunoreactive somatostatin released from hypothalamus ($V_0/V_e = 0.48$) and spinal cord ($V_0/V_e = 0.47$) on Sephadex G-25 (fine grade) gel chromatography

V_0 = void volume; the column was 90 cm $\times$ 1.6 cm; V_0/V_e for synthetic somatostatin = 0.48.

vitro was indicated by the demonstration of linear O_2 consumption over 6 h ($r = 0.954$, $P < 0.001$), with a consumption rate of $212.9 \pm 14.5 \mu\text{mol}$ of O_2/h per g (mean $\pm$ S.E.M., $n = 3$).

(2) Theophylline at 0.5–10.0 mM caused a dose-dependent increase in the release of rat somatostatin (Fig. 7). For all further experiments a concentration of 2.5 mM-theophylline was chosen, a value closely corresponding to the concentration giving rise to the half-maximal response.

(3) Synthetic somatostatin at 0.05–1.00 ng/ml caused a dose-dependent inhibition of 2.5 mM-theophylline-stimulated rat somatotropin release, allowing a dose-response curve to be constructed (Fig. 8).

(4) Freeze-dried 1 M-acetic acid eluates from anti-somatostatin affinity columns (resuspended in perfusion buffer) inhibited 2.5 mM-theophylline-stimulated rat somatotropin release in all instances. Logit-log transformation of results obtained from studying the inhibitory effect of serial dilutions of released immunoreactive somatostatin allowed comparison with a similarly transformed inhibition curve resulting from dilutions of synthetic somatostatin standard. Serial dilutions of somatostatin immunoreactivity released from all four tissues studied

Fig. 5. Elution profiles of immunoreactive somatostatin released from suspensions of hypothalamic synaptosomes ($V_0/V_e = 0.46$) and cortical synaptosomes ($V_0/V_e = 0.46$) on Sephadex G-25 (fine grade) gel chromatography V_0 = void volume; the column was 94 cm $\times$ 1.6 cm; V_0/V_e for synthetic somatostatin = 0.47.

Fig. 6. Elution profiles of immunoreactive somatostatin released from suspensions of thalamic synaptosomes ($V_0/V_e = 0.46$) and striatal synaptosomes ($V_0/V_e = 0.45$) on Sephadex G-25 (fine grade) gel chromatography V_0 = void volume; the column was 94 cm $\times$ 1.6 cm; V_0/V_e for synthetic somatostatin = 0.47.

Fig. 7. Effect of increasing concentrations of theophylline on immunoreactive somatotropin release from perfused hemipituitaries

Results are expressed as percentages of the basal value, where the basal value is the mean for four fractions before stimulus, and the response value is the mean for four fractions after stimulus (values shown are means $\pm$ S.E.M. for the number of experiments indicated in parentheses).

Fig. 8. Inhibition of immunoreactive somatotropin release from perfused hemipituitaries by increasing concentrations of synthetic somatostatin

Results are expressed as means $\pm$ S.E.M. for the number of experiments indicated in parentheses. (See the text for calculation of percentage inhibition.)

showed parallelism with decreasing amounts of synthetic somatostatin (Figs. 9 and 10).

Discussion

Inhibition curves generated in the immunoassay by somatostatin immunoreactivity released from rat hypothalamus, spinal cord and hypothalamic, cortical, thalamic and striatal synaptosomes were

Fig. 9. Logit-log plot of somatotropin inhibitory response produced by serial dilutions of immunoreactive somatostatin released from hypothalamus ($\square$) ($b = 2.604$, $r = 0.658$, $n = 14$) and spinal cord ($\blacktriangledown$) ($b = 2.845$, $r = 0.885$, $n = 11$), showing parallelism to inhibitory response produced by decreasing concentrations of synthetic somatostatin ($\circ$) ($b = 2.657$, $r = 0.867$, $n = 29$)

See the text for calculation of logit (inhibitory response).

Fig. 10. Logit-log plot of somatotropin inhibitory response produced by serial dilutions of immunoreactive somatostatin released from suspensions of hypothalamic synaptosomes ($\bullet$) ($b = 2.566$, $r = 0.731$, $n = 12$) and cortical synaptosomes ($\blacktriangledown$) ($b = 2.466$, $r = 0.958$, $n = 11$), showing parallelism to inhibitory response produced by decreasing concentrations of synthetic somatostatin ($\circ$) ($b = 2.657$, $r = 0.867$, $n = 29$)

See the text for calculation of logit (inhibitory response).

Table 1. *Immunoaffinity chromatography of synthetic somatostatin and released somatostatin immunoreactivity*
 Percentage recovery from the columns was calculated from the amount of material applied to the column and the total eluted with both phosphate-buffered saline and 1 M-acetic acid. The amount of synthetic somatostatin standard or sample immunoreactivity (released immunoreactive material from hypothalamus, spinal cord and hypothalamic and cortical synaptosomes) eluted with phosphate-buffered saline and with acetic acid was expressed as a percentage of total material eluted from the column. Results are expressed as means $\pm$ S.E.M., $n = 3$.

	Somatostatin standard	Hypothalamus	Spinal cord	Hypothalamic synaptosomes	Cortical synaptosomes
Anti-somatostatin column					
Recovery (%)	86.6 $\pm$ 3.2	85.5 $\pm$ 1.9	90.1 $\pm$ 0.8	90.7 $\pm$ 4.6	88.2 $\pm$ 3.1
Eluted with phosphate-buffered saline (%)	14.4 $\pm$ 6.6	13.1 $\pm$ 3.5	5.8 $\pm$ 1.7	14.6 $\pm$ 4.9	8.0 $\pm$ 3.0
Eluted with acetic acid (%)	85.6 $\pm$ 6.6	86.9 $\pm$ 3.5	94.3 $\pm$ 1.7	85.4 $\pm$ 4.9	92.0 $\pm$ 3.0
Non-immune-rabbit-serum column					
Recovery (%)	84.3 $\pm$ 3.1	85.2 $\pm$ 2.0	81.7 $\pm$ 1.9	83.5 $\pm$ 3.8	83.3 $\pm$ 0.9
Eluted with phosphate-buffered saline (%)	83.7 $\pm$ 3.7	85.2 $\pm$ 5.0	87.5 $\pm$ 0.9	92.3 $\pm$ 1.0	90.0 $\pm$ 6.9
Eluted with acetic acid (%)	16.3 $\pm$ 3.7	14.8 $\pm$ 5.0	12.5 $\pm$ 0.9	7.7 $\pm$ 1.0	10.0 $\pm$ 6.9

parallel to the inhibition curve produced by synthetic somatostatin standard. Somatostatin immunoreactivity released from all tissue areas studied co-eluted with synthetic tetradecapeptide on Sephadex G-25 (fine grade) gel chromatography. The major portion (more than 85%) of standard or sample immunoreactivity released from hypothalamus, spinal cord, hypothalamic and cortical synaptosomes, bound to Sepharose-anti-somatostatin-serum immunoaffinity columns, whereas very little of the material bound to Sepharose-non-immune-rabbit-serum control columns. These observations suggest that the somatostatin released from a number of rat brain preparations *in vitro* is immunologically similar to synthetic tetradecapeptide somatostatin. In three fairly recent studies (Maeda & Frohman, 1980; Terry *et al.*, 1980; Richardson *et al.*, 1980), serial dilutions of medium somatostatin immunoreactivity from incubated or perfused hypothalamic fragments were shown to be parallel in activity to dilutions of synthetic somatostatin standards, confirming the immunological similarity of somatostatin released from rat hypothalamus *in vitro* to the synthetic tetradecapeptide. We have now confirmed the release of immunologically reactive somatostatin from extra-hypothalamic areas.

The chromatographic characteristics of central-nervous-system immunoreactive somatostatin have been studied extensively. There is considerable evidence that hypothalamic and extrahypothalamic tissues contain somatostatin-like substances having molecular weights greater than that of the tetradecapeptide. A fairly recent report has described the existence of three molecular-weight species of somatostatin-like immunoreactivity separated from acetic acid extracts of rat hypothalamus (Spiess &

Vale, 1980); Böhlen *et al.* (1980) have isolated a biologically active somatostatin containing 28 amino acid residues from ovine hypothalami, as well as a shorter, 25-amino-acid, peptide; and Zingg & Patel (1979) have reported the existence of two high-molecular-weight forms of immunoreactive somatostatin in rat median eminence and neurohypophysis in addition to tetradecapeptide somatostatin. Rorstadt *et al.* (1979) have shown that immunoaffinity-purified immunoreactive somatostatin from four rat brain areas (median eminence, anterior hypothalamic-preoptic area, amygdala and parietal cortex) eluted as four peaks on Sephadex G-25 (fine grade) gel chromatography. In addition, each peak possessed biological activity by virtue of its ability to inhibit somatotropin release from cultured rat anterior-pituitary cells. There seems little doubt, therefore, that a number of different molecular-weight forms of somatostatin-like immunoreactivity exist which have similar immunological and biological activities but different physicochemical properties.

The chromatographic characteristics of the released material are less well-defined. The high-molecular-weight forms of somatostatin-like immunoreactivity described by Zingg & Patel (1979) were released into the medium from incubated rat median eminence and neurohypophysis *in vitro*. Richardson *et al.* (1980) demonstrated, by Sephadex G-25 chromatographic studies, that although the bulk of somatostatin immunoreactivity released from incubated hypothalamic fragments co-eluted with synthetic tetradecapeptide somatostatin, two further peaks of immunoreactivity were present which eluted in advance of synthetic somatostatin. High-pressure liquid chromatography confirmed that the third peak co-eluted with synthetic

tetradecapeptide. By contrast, in the present studies and in those of Maeda & Frohman (1980), all the recovered immunoreactive somatostatin co-eluted with the synthetic tetradecapeptide. It would appear, therefore, that central-nervous-system *extracts* contain multiple molecular-weight species, whereas *released* material is predominantly a single species with the former perhaps representing peptide at various stages of processing from precursor. Inability of the antiserum used to recognize high-molecular-weight forms is another possibility. The antiserum used in our studies shows good cross-reaction with des-Ala¹-Gly²-somatostatin, [Tyr¹]-somatostatin and cyclic-(4–13)-somatostatin, but no cross-reactivity with biologically inactive D-Phe⁶-, D-Phe⁷-, Phe⁸- or Pro⁸-substituted somatostatin (Berelowitz *et al.*, 1978c), suggesting a locus of cross-reactivity away from the N-terminus and related to amino acids in positions 6–8, regarded as biologically important for somatotropin inhibition.

The biological activity of the released immunoreactive somatostatin has been assessed by the ability of the material to inhibit theophylline-stimulated somatotropin release from the anterior pituitary. A number of advantages have been claimed for perfusion of anterior pituitaries, and these include: (1) the ability to apply a sequence of tests to a single gland so that each gland acts as its own control; (2) the continuous turnover of medium, which thus minimizes the effects of accumulation of secretory or metabolic products and decreases the breakdown of substances secreted by the gland or administered to evoke a response; and (3) the demonstration that perfused rat pituitaries remain responsive to stimuli for at least 6 h, accompanied by linear somatotropin synthesis for 8 h (Dowd *et al.*, 1975; Stachura & Frohman, 1974). To ensure that the biological activity of only immunoreactive somatostatin was being assessed, acetic acid eluates from Sepharose–somatostatin-antiserum affinity columns were freeze-dried, re-suspended in perfusion medium and tested in the system. The demonstration that the immunoreactive material from hypothalamus, spinal cord and hypothalamic and cortical synaptosomes inhibited somatotropin release in a parallel dose-related manner indicates the biological similarity to synthetic somatostatin, and demonstrates that even though the peptide may have a neuromodulator function in the extrahypothalamic nervous system, it nevertheless has the biological potential to inhibit somatotropin secretion. It should once again be emphasized, however, that despite immunological and biological similarity between the material released from these hypothalamic and extrahypothalamic areas and synthetic tetradecapeptide somatostatin, confirmation of identity awaits purification and sequencing of the polypeptide.

References

- Bennett, G. W., Edwardson, J. A., Marcano de Cotte, D., Berelowitz, M., Pimstone, B. L. & Kronheim, S. (1979) *J. Neurochem.* **32**, 1127–1130
- Berelowitz, M., Kronheim, S., Pimstone, B. & Sheppard, M. (1978a) *J. Neurochem.* **31**, 1537–1539
- Berelowitz, M., Hudson, A., Pimstone, B., Kronheim, S. & Bennett, G. W. (1978b) *J. Neurochem.* **31**, 751–753
- Berelowitz, M., Kronheim, S., Pimstone, B. & Shapiro, B. (1978c) *J. Clin. Invest.* **61**, 1410–1414
- Böhlen, P., Brazeau, P., Benoit, R., Ling, N., Esch, F. & Guillemin, R. (1980) *Biochem. Biophys. Res. Commun.* **96**, 725–734
- Carlson, H. E., Mariz, I. K. & Daughaday, W. H. (1974) *Endocrinology* **94**, 1709–1713
- Coy, D. H., Coy, E. J., Arimura, A. & Schally, A. V. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1267–1273
- Dowd, A. J., Barofsky, A.-L., Chaudhuri, N., Lloyd, C. W. & Weisz, J. (1975) *Endocrinology* **96**, 243–252
- Feldman, H. & Rodbard, D. (1971) in *Principles of Competitive Protein Binding Assays* (Odell, W. D. & Daughaday, W. H., eds.), pp. 158–203. J. P. Lippincott Co., Philadelphia
- Iversen, L. L., Iversen, S. D., Bloom, F., Douglas, C., Brown, M. & Vale, W. (1978) *Nature (London)* **273**, 161–163
- Krieger, D. T. & Martin, J. B. (1981) *N. Engl. J. Med.* **304**, 876–885
- Kronheim, S., Berelowitz, M. & Pimstone, B. L. (1976) *Clin. Endocrinol.* **5**, 619–630
- Maeda, K. & Frohman, L. A. (1980) *Endocrinology* **106**, 1837–1842
- Negro-Vilar, A., Ojeda, S. R., Arimura, A. & McCann, S. M. (1978) *Life Sci.* **23**, 1493–1497
- Patel, Y. C., Zingg, H. H. & Dreifuss, J. J. (1977) *Nature (London)* **267**, 852–853
- Richardson, S. B., Hollander, C. S., D'Eletto, R., Greenleaf, P. W. & Thaw, C. (1980) *Endocrinology* **107**, 122–129
- Rivier, J. E. F. (1974) *J. Am. Chem. Soc.* **96**, 2986–2992
- Rorstad, O. P., Epelbaum, J., Brazeau, P. & Martin, J. B. (1979) *Endocrinology* **105**, 1083–1092
- Sheppard, M., Kronheim, S., Adams, C. & Pimstone, B. (1979) *Neurosci. Lett.* **15**, 65–70
- Sheppard, M. C. (1981) in *Neuropeptides: Biochemical and Physiological Studies* (Millar, R. P., ed.), pp. 97–103, Churchill–Livingstone, Edinburgh
- Spiess, J. & Vale, W. (1980) *Biochemistry* **19**, 2861–2866
- Stachura, M. E. & Frohman, L. A. (1974) *Endocrinology* **94**, 701–712
- Terry, L. C., Rorstad, O. P. & Martin, J. B. (1980) *Endocrinology* **107**, 794–800
- Yalow, R. S. & Berson, S. A. (1971) in *Principles of Competitive Protein Binding Assays* (Odell, W. D. & Daughaday, W. H., eds.), pp. 374–400, J. P. Lippincott Co., Philadelphia
- Yamashiro, D. & Li, C. H. (1973) *Biochem. Biophys. Res. Commun.* **54**, 882–888
- Zingg, H. H. & Patel, Y. C. (1979) *Biochem. Biophys. Res. Commun.* **90**, 466–472