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Purification and characterization of human neuropeptide Y from adrenal-medullary phaeochromocytoma tissue

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Human neuropeptide Y was isolated from acid extracts of adrenal-medullary phaeochromocytoma tissue. After $(NH_4)_2SO_4$ fractionation, the neuropeptide Y-like immunoreactivity was purified from the resolubilized 80%-saturation-$(NH_4)_2SO_4$ peptide-rich precipitate, by gel filtration, cation-exchange chromatography and reverse-phase high-pressure liquid chromatography. Amino acid analysis of the peptide revealed a composition almost identical with that of the pig peptide, the exception being the loss of one leucine residue and its replacement with methionine. Tryptic digestion of the peptide and subsequent amino acid analysis of the fragments further confirmed the identity of the peptide. Carboxypeptidase Y digestion of the (1–19)-peptide tryptic fragment has shown the methionine to be located at position 17 in human neuropeptide Y.

A number of biologically active peptides have been found stored with classical transmitters in neurons of the central and peripheral nervous systems (Hökfelt *et al.*, 1980). Several of these peptides, which may function as neurotransmitters or hormones, have been reported in the adrenal medulla (Hökfelt *et al.*, 1980. Corder *et al.*, 1982*a*). By using immunohistochemical techniques one such peptide, avian pancreatic polypeptide (APP), was observed with a widespread distribution in both the brain and periphery, including a subpopulation of adrenal-medullary cells of the cat and rat (Lundberg *et al.*, 1980). In fact many of the cells containing an APP-like immunoreactive substance were found to be catecholaminergic, including in the peripheral nervous system, sympathetic-ganglion cells of the superior cervical, stellate and coeliac ganglia as well as the APP-positive cells of the adrenal medulla.

Although APP was isolated from chicken pancreas and structurally characterized in 1975 (Kimmel *et al.*, 1975), the exact nature of the APP-like immunoreactive material demonstrated in rats and cats (Lundberg *et al.*, 1980) was not known. However, the identity of the APP-like immunoreactivity has become clearer with the subsequent isolation of two structurally related peptides: neuropeptide Y (NPY) and peptide YY (PYY), from pig brain and gut respectively (Tatemoto, 1982*a*,*b*). These peptides, NPY, PYY and APP, are all 36 amino acid residues in length with a *C*-terminal tyrosine amide and possess a high degree of sequence homology: PYY and NPY have identical residues in 25 positions (Tatemoto, 1982*b*), and NPY and APP share 20 identical residues (Fig. 1). Structural homology also exists, but to a lesser degree, between NPY and the human (Fig. 1), bovine and pig pancreatic polypeptide hormones (Tatemoto, 1982*b*).

Recent studies have shown the presence of NPY-like immunoreactivity in peripheral noradrenergic neurons, where it is located in the same population of nerve cells to those staining positively with antiserum raised against APP (Lundberg *et al.*, 1982). Furthermore the anti-APP serum used previously (Lundberg *et al.*, 1980) was found to cross-react with NPY (Lundberg *et al.*, 1982), thus indicating that the observed APP-like immunoreactivity is probably due to the presence of the structurally related NPY in these neurons. In the present paper we provide further evidence to support the concept that APP-like immunoreac-

Abbreviations used: APP, avian pancreatic polypeptide; NPY, neuropeptide Y; PYY, peptide YY; h.p.l.c., high-pressure liquid chromatography; ODS-silica, octadecylsilyl-silica.

	1	5	10	15	20	25	30	35
NPY	Y	P S K P D N P G E D A P A E D L A R Y Y S A L R H Y I N L I T R Q R Y[a]						
APP	G	P S Q P T Y P G D D A P V E D L I R F Y D N L Q Q Y L N V V T R H R Y[a]						
HPP	A	P L E P V Y P G D N A T P E Q M A Q Y A A D L R R Y I N M L T R P R Y[a]						

Fig. 1. *Amino acid sequences of NPY and two structurally related peptides*
The primary structures of the following 36-residue peptides are illustrated: neuropeptide Y (NPY) (Tatemoto, 1982*b*), avian pancreatic polypeptide (APP) (Kimmel *et al.*, 1975) and human pancreatic polypeptide (HPP) (Lonovics *et al.*, 1981). The one-letter notation used is that recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (1969). Amidation of the *C*-terminal amino acids is indicated by [a].

tivity found in peripheral noradrenergic neurons and adrenal medulla is due to the presence of the brain peptide NPY.

We have previously reported the characterization of the enkephalin and somatostatin from human adrenal-medullary phaeochromocytoma tissue (Clement-Jones *et al.*, 1980; Corder *et al.*, 1982*b*), as well as the identification of a number of large-molecular-mass forms of both enkephalin and somatostatin (Clement-Jones *et al.*, 1982; Corder & Lowry, 1982). Subsequently we have pooled the acid extracts from a large number of these tumours in order to increase the starting material from which we are able to isolate new peptides. Recently we observed the presence of NPY-like immunoreactivity in this fractionated extract after Sephadex G-100 gel-filtration chromatography. Here we report the purification and characterization of NPY from pooled extracts of 12 human adrenal-medullary tumours.

Materials and methods

Materials

Fragments of adrenal-medullary tumours from 12 patients were collected fresh at surgery and either stored at −70°C until extracted or extracted immediately and the fractionated extract stored at −20°C. Only tumours identified histologically as phaeochromocytoma were included in the purification.

Sephadex G-100, Sephadex G-75 (superfine grade) and CM-Sepharose CL-6B were from Pharmacia, Hounslow, Middx., U.K. The Gilson system 42 h.p.l.c. equipment (purchased from Anachem, Luton, Beds., U.K.) was linked to a Uvikon 740 LC detector (Kontron Instruments, St. Albans, Herts., U.K.), for monitoring u.v. absorption, and fluorescence detection was performed with a Pye–Unicam LC-FL detector (Pye–Unicam, Cambridge, U.K.). Ultrasphere h.p.l.c. columns manufactured by Altex Scientific, Berkeley, CA, U.S.A., were purchased from Anachem. Bulk 5 μm Hypersil ODS-silica was from Shandon Southern Products, Runcorn, Cheshire, U.K. The Shimadzu UV-150-02 double-beam spectrophotometer was from Seizakusho, Kyoto, Japan.

H.p.l.c.-grade solvents and trifluoroacetic acid were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.). Amino acid standards for amino acid analysis were from Calbiochem (Bishop's Stortford, Herts., U.K.). Carboxypeptidase B (EC 3.4.17.2), *o*-phthaldialdehyde, and tyrosine amide were from Sigma Chemical Co. (Poole, Dorset, U.K.). Carboxypeptidase Y and HCl (constant-boiling Sequanal grade) were from Pierce and Warriner, Chester, U.K. 1-Chloro-4-phenyl-3-tosylamidobutan-2-one-treated trypsin (EC 3.4.21.4) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. All other reagents were AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.).

Extraction and fractionation

Fragments of adrenal-medullary tumours were obtained at surgery from 12 patients (total wet wt. 1607g). The tumour tissue from each patient was extracted separately by homogenization in 10vol. of 0.1M-HCl. The homogenate was then heated at 80°C for 15min to inactivate proteinases, before the addition of $(NH_4)_2SO_4$ to 10% saturation and centrifugation at 18000*g* for 30min at 4°C. After centrifugation the supernatant was retained and the precipitated tissue debris was discarded. The $(NH_4)_2SO_4$ concentration in the decanted supernatant was increased to 80% saturation, and the mixture was then centrifuged as above for a further 60min. The peptide-rich precipitates obtained from the second centrifugation were stored at −20°C until being subjected to further purification. Samples from these precipitates and from the 80%-saturation-$(NH_4)_2SO_4$ supernatants were subjected to radioimmunoassay for enkephalin and somatostatin, and also to some preliminary gel-filtration investigations (Clement-Jones *et al.*, 1982; Corder *et al.*, 1982*b*). The total weight of precipitate after centrifugation with 80%-saturation $(NH_4)_2SO_4$ was 25g. This was pooled and reconstituted in 600ml of 0.27M-formic acid, filtered con-

secutively through 8 μm-, 5 μm- and 1.2 μm-pore-size polycarbonate filters (Millipore, London N.W.10, U.K.). The resultant filtrate was subjected to Sephadex G-100 gel-filtration chromatography.

Chromatography

The Sephadex G-100 gel-filtration column was used at room temperature, and CM-Sepharose CL-6B ion-exchange and Sephadex G-75 gel-filtration columns were used at 4°C; the fractions from all chromatographic procedures were collected at 4°C and stored at −20°C between each purification step. Samples from chromatographic fractions were measured for NPY-like immunoreactivity by using a radioimmunoassay as described below, and monitored for absorbance at 280nm.

Sephadex G-100 gel-filtration chromatography (*Fig. 2*). The fractionated peptide was loaded on to a column (10cm × 95cm) of Sephadex G-100 and eluted with 0.027M-formic acid at a flow rate of 100ml/h; 90min fractions (150ml) were collected. Fractions containing the NPY-like immunoreactivity as well as large-molecular-mass enkephalin-like and somatostatin-like immunoreactivity (4900–6800ml) were pooled. The pH was adjusted to 4.7 with conc. NH_3 solution (35%, sp.gr. 0.880), which caused slight precipitation. The solution was clarified by centrifugation at 2000*g* for 1h at 4°C.

CM-Sepharose CL-6B ion-exchange chromatography (*Fig. 3*). The pooled fractions from Sephadex G-100 gel filtration were loaded on to a column (2.5 × 28.5cm) of CM-Sepharose CL-6B equilibrated in 0.02M-ammonium acetate (pH4.7, adjusted by dropwise addition of acetic acid). After the loading the column was washed with 200ml of 0.02M-ammonium acetate and then eluted with a linear salt gradient from 0.02M- to 0.5M-ammonium acetate at pH4.7 (total gradient volume 2 litres), and finally washed with 300ml of 0.5M-ammonium acetate containing 20% (v/v) acetonitrile. A flow rate of 25ml/h was employed, and 25min (10.5ml) fractions were collected from the beginning of the salt gradient.

Sephadex G-75 gel-filtration chromatography (*Fig. 4*). The fractions containing the NPY-like immunoreactive material eluted from the CM-Sepharose between 1605 and 1738ml of the salt gradient were freeze-dried. The resultant residue was reconstituted in 10ml of 0.05M-HCl and loaded on to a column (2.5cm × 88.5cm) of Sephadex G-75, developed at a flow rate of 6ml/h, and 90min (9ml) fractions were collected.

H.p.l.c. Final purification of the NPY-like peptide was achieved in two h.p.l.c. steps. Firstly, the three fractions from Sephadex G-75 gel filtration, containing the NPY-like material, were pumped on to a semi-preparative 1cm × 15cm column of 5 μm Ultrasphere octylsilyl-silica; this was eluted at 3ml/min, with a 30min linear gradient of 24–60% (v/v) acetonitrile containing 0.1% trifluoroacetic acid, 0.5min fractions were collected and absorbance was measured at 280nm (Fig. 5). One fraction was taken (17.8–18.3min) from the peak of NPY-like immunoreactivity eluted between 16.8 and 18.8min, and diluted with 2vol. of 0.1% trifluoroacetic acid and re-chromatographed on an analytical 4.6mm × 150mm 5 μm Ultrasphere ODS-silica column developed at 1ml/min, with a 60min linear gradient of 0–50% (v/v) propan-2-ol containing 0.1% trifluoroacetic acid; absorption was monitored at 280nm. This second column and solvent system was also used to resolve the tryptic fragments of the NPY-like peptide; see below and Fig. 6, where the elution position of the purified NPY is indicated.

Radioimmunoassay

The NPY radioimmunoassay was carried out in 0.05M-phosphate buffer, pH7.4 (0.04M-Na_2HPO_4/0.01M-NaH_2PO_4), containing 0.5% bovine serum albumin. The antiserum was raised in rabbits against pig NPY coupled to bovine serum albumin by using carbodi-imide. Standards or samples were incubated in assay buffer with antibody (final dilution 1:16000) and ^{125}I-NPY for 48h at 4°C; free ^{125}I-NPY was then separated from bound ^{125}I-NPY by using 1ml of 2.25% charcoal suspension containing 10% human blood-bank plasma in assay buffer.

Tryptic digestion

A sample 200 μl (2.5nmol) of peptide was taken from the peak fraction obtained on analytical h.p.l.c. and diluted in 600 μl of 0.1M-$NaHCO_3$. To this peptide solution 100 μl of 0.05M-$NaHCO_3$ containing 2 μg of 1-chloro-4-phenyl-3-tosylamidobutan-2-one-treated trypsin was added; after incubation for 2.5h at 37°C the digestion was terminated by the addition of 10 μl of trifluoroacetic acid. The resultant peptide fragments were resolved by analytical h.p.l.c. of the mixture, under conditions identical with those used for the final purification step (Fig. 6); absorption was monitored at 215nm.

Amino acid analysis

Peptide samples (2–5nmol) (see the Results and discussion section) were hydrolysed with 200 μl of constant-boiling HCl, in evacuated Pyrex tubes, for 20h at 120°C. After the hydrolysates had been dried down, under reduced pressure, to remove the HCl, each sample was reconstituted in 1.3ml of 0.01M-HCl, 50 μl was retained for analysis by h.p.l.c., and 1ml of the reconstituted hydrolysate

was analysed on a Jeol JLC-6AH automatic amino acid analyser (Japanese Electric and Optical Co., Tokyo, Japan), which employs a ninhydrin detection system.

Amino acid analysis by h.p.l.c. was based on the method of Jones *et al.* (1981), pre-column labelling with *o*-phthaldialdehyde being followed by reverse-phase h.p.l.c. separation and fluorescence detection of the amino acid derivatives.

Essentially 20 μl of amino acid standards or hydrolysates was treated with 20 μl of derivative-forming solution (25 mg of *o*-phthaldialdehyde, 0.65 ml of methanol, 50 μl of 2-mercaptoethanol and 5.5 ml of 0.4 M-potassium borate, pH 9.5). After 0.5 min 80 μl of 0.4 M-NaH_2PO_4 was added, the solution vortex-mixed and 100 μl injected on to the h.p.l.c. system. Chromatographic separation of the fluorescent amino acid derivatives was performed on a 4.6 mm × 250 mm column of 5 μm Ultrasphere ODS-silica fitted with a 4.6 mm × 50 mm pre-column packed with 5 μm Hypersil ODS-silica. Gradient elution at ambient temperature and flow rate of 1 ml/min was performed with the use of solvent A (10% methanol, 1% tetrahydrofuran, 89% 0.05 M-sodium acetate, pH 5.9) and solvent B (80% methanol, 20% 0.05 M-sodium acetate, pH 5.9).

Carboxypeptidase Y hydrolysis

A sample (100 μl) of the fraction containing tryptic peptide T1 (Fig. 6) was mixed with 20 μl of 0.06 M-NaOH and 70 μl of 0.1 M-sodium acetate, pH 5.9. To this solution 5 μg of carboxypeptidase Y was added and the mixture incubated at room temperature. Samples (20 μl) were taken for blank measurement and identification of released amino acids, over a time course (Table 3), by using the *o*-phthaldialdehyde pre-column labelling and h.p.l.c. separation as described above.

Carboxypeptidase B hydrolysis

After tryptic digestion of the purified NPY-like peptide the fragments were resolved by h.p.l.c. A sample (50 μl) of the material eluted without retention from the ODS-silica column (3.8–5 min; Fig. 6) was taken and mixed with 12.5 μl of 0.06 M-NaOH, 50 μl of 0.1 M-$NaHCO_3$ and 1 μg of carboxypeptidase B, and incubated for 90 min at room temperature. Samples (20 μl) were taken at the start and after 90 min and subjected to h.p.l.c. amino acid analysis.

Detection of tyrosine amide

Tyrosine amide was measured by u.v. absorption at 280 nm, after h.p.l.c. The same ODS-silica columns as those used for h.p.l.c. amino acid analysis were used in the chromatographic separation of tyrosine amide and tyrosine. Samples or standards were dissolved in 0.05 M-sodium acetate, pH 5.9, which was also used as elution buffer at a flow rate of 1 ml/min; 3 min after injection the system was developed with a 30 min linear gradient from 0 to 16% (v/v) acetonitrile.

Results and discussion

Extraction and purification

Neither the efficiency of the acid extraction nor the losses of NPY-like immunoreactivity during $(NH_4)_2SO_4$ fractionation were monitored, since the initial steps were carried out with the aim of purifying large-molecular-mass pro-enkephalin and pro-somatostatin polypeptides. The recoveries of the NPY-like immunoreactive peptide in all subsequent purification steps were greater than 90%.

The NPY-like immunoreactivity was eluted in a single peak immediately before the salt on Sephadex G-100 gel filtration (Fig. 2), characteristic of a peptide with a molecular mass of 4200 Da. In order to include the other peptide immunoreactivities we were studying, the pooled fractions from size-exclusion chromatography that were subjected to CM-Sepharose ion-exchange chromatography incorporated a broader elution volume (4.9–6.8 litres) than just the NPY-like immunoreactivity. A single peak of NPY-like immunoreactivity was obtained from CM-Sepharose ion-exchange chromatography (Fig. 3), running in the latter part of the salt gradient, and displaying the properties of a basic peptide. The NPY-containing fractions were pooled and freeze-dried, and a second gel-filtration step with Sephadex G-75 was performed (Fig. 4) to produce a sample composed only of 4000–5000 Da peptides suitable for h.p.l.c. purification.

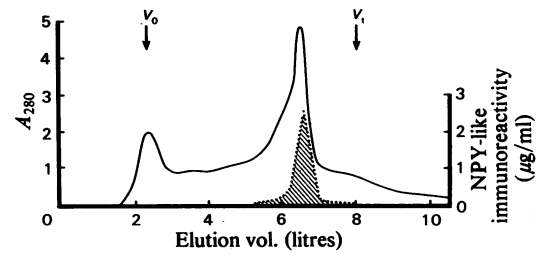

Fig. 2. *Gel-filtration chromatography on Sephadex G-100 of the re-solubilized 80%-saturation-$(NH_4)_2SO_4$ peptide precipitate*

The Figure shows elution of the NPY-like immunoreactivity (▨), from a column (10 cm × 95 cm) of Sephadex G-100. Eluent buffer (0.027 M-formic acid) was pumped at 100 ml/h, and 90 min fractions were collected at 4°C. ——, A_{280}. V_0, Void volume; V_t, total volume.

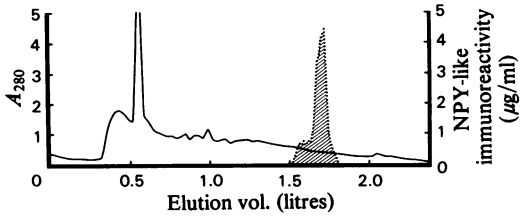

Fig. 3. *Cation-exchange chromatography on CM-Sepharose of the peptides eluted from Sephadex G-100*
The Sephadex G-100 fractions from 4.9 to 6.8 litres were pooled and the pH was adjusted to 4.7. After this preparation had been loaded on to the column (2.5cm × 28.5cm) of CM-Sepharose, bound material was eluted with a linear salt gradient from 0.02- to 0.5M-ammonium acetate, pH 4.7, as described in the Materials and methods section. ▨, NPY-like immunoreactivity; ——, A_{280}.

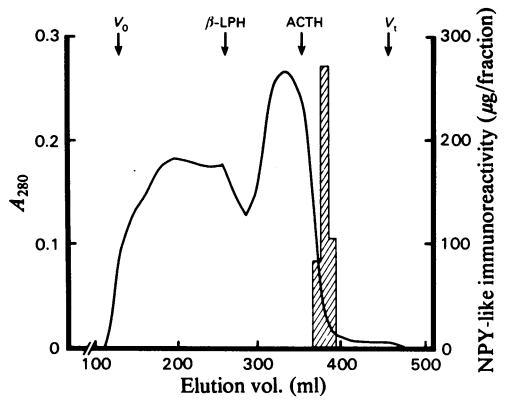

Fig. 4 *Fractionation on Sephadex G-75 of the NPY-like peptides obtained from CM-Sepharose*
NPY-like peptides eluted from CM-Sepharose between 1.6 and 1.74 litres were freeze-dried. After reconstitution in 10ml of 0.05M-HCl the material was chromatographed on a column (2.5cm × 88.5cm) of Sephadex G-75, eluted with 0.27M-formic acid. ▨, NPY-like immunoreactivity; ——, A_{280}. The elution positions of the M_r markers human β-lipotropin (β-LPH) and corticotropin (ACTH) are indicated. V_0, Void volume; V_t, total volume.

Semi-preparative h.p.l.c. on Ultrasphere octylsilyl-silica produced one main peak of NPY-like immunoreactivity (Fig. 5) eluted between 16.8 and 18.8min at 45% acetonitrile. The absorption measured at 280nm showed a skewed peak, which did not correlate with the shape of the peak of immunoreactivity (Fig. 5). Amino acid analysis of the fractions showed considerable contamination

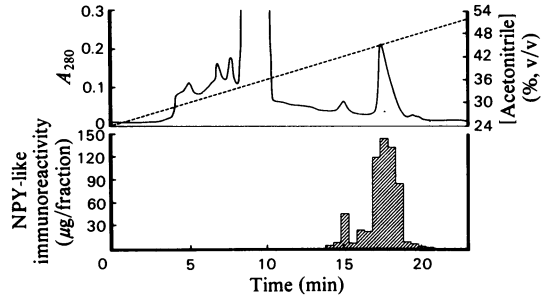

Fig. 5. *Semi-preparative h.p.l.c. of the NPY-like peptide from Sephadex G-75*
The fractions containing the NPY-like immunoreactivity from Sephadex G-75 were pumped on to a column (1cm × 15cm) of 5μm Ultrasphere octylsilyl-silica. This was developed at ambient temperature and flow rate of 3ml/min with a 30min linear gradient of 24–60% acetonitrile containing 0.1% trifluoroacetic acid; 0.5min fractions were collected. ▨, NPY-like immunoreactivity; ——, A_{280}; – – – –, acetonitrile gradient.

of the first two fractions, 16.8–17.8min, with a tyrosine-containing peptide, which accounted for the shape of the u.v. absorption trace. However, it was possible to resolve the NPY-like peptide from the tyrosine-containing contaminant by analytical h.p.l.c. on a 4.6mm × 150mm column of 5μm Ultrasphere ODS-silica with a linear gradient of 0–50% propan-2-ol containing 0.1% trifluoroacetic acid. By using this procedure it was possible to purify further the third fraction in the peak (17.8–18.3min; Fig. 5), which was only slightly contaminated, producing a homogeneous peptide suitable for chemical characterization. The h.p.l.c. purification finally yielded approx. 500μg of NPY from the 1607g of starting tissue.

Chemical characterization

The final h.p.l.c. purification step resulted in a pure peptide with an analysis closely resembling that of pig NPY (Table 1). The only difference between the human adrenal-medullary peptide and the pig brain peptide was the loss of one leucine residue per molecule and its replacement with a methionine residue.

The peptide fragments obtained on tryptic digestion were resolved by h.p.l.c. (Fig. 6), this produced a profile of considerable similarity to that published for pig NPY (Tatemoto, 1982*b*). Amino acid analysis of the tryptic peptides identified them as peptides T1 (amino acid residues 1–19), T2 (residues 20–25) and T3 (residues 26–33) (Fig. 6 and Table 2). Interestingly, the elution positions of peptides T1 and T2 are the reverse of those found

Table 1. *Amino acid analysis of the purified human NPY-like peptide*

Peptides were hydrolysed as described in the Materials and methods section. The values for serine and threonine are corrected for 30% destruction during hydrolysis. The compositions are based on the assumption of four arginine residues/molecule. Results were obtained by using (*a*) a Jeol JLC-6AH automatic analyser with ninhydrin detection and (*b*) pre-column labelling with *o*-phthaldialdehyde and h.p.l.c. separation of the resultant amino acid derivatives and fluorescence detection. The composition of pig NPY is also indicated. Cysteine, valine and phenylalanine were not detected.

	Amino acid composition (mol/mol of peptide)		
	Human NPY-like peptide		
	(*a*)	(*b*)	Pig NPY
Asp	4.95	5.45	5
Thr	1.00	1.01	1
Ser	2.28	1.39	2
Glu	3.23	3.24	3
Gly	0.98	0.86	1
Ala	4.25	3.91	4
Met	0.98	0.68	0
Ile	2.05	1.90	2
Leu	2.07	1.85	3
Tyr	4.92	4.60	5
His	0.87	0.77	1
Lys	0.96	1.29	1
Arg	4.00	4.00	4
Pro	3.98	–	4

by Tatemoto (1982*b*). However, the decrease in relative hydrophobicity of peptide T1 compared with peptide T2 in the h.p.l.c. system used in the present work can be accounted for by the use of a different reverse-phase column packing and eluting solvent, and the substitution of leucine in peptide T1 by the less hydrophobic methionine residue (Table 2).

The amino acid analyses obtained for the tryptic fragments T2 and T3 (Table 2) were identical with those for the pig peptide (Tatemoto, 1982*b*). The presence of a methionine residue in peptide T1 and the absence of leucine suggested a straightforward exchange of one for the other in human adrenal-medullary NPY. This was confirmed by time-course hydrolysis of peptide T1 with carboxypeptidase Y, which showed the methionine residue to be at position 17 in the human peptide (Table 3). Final confirmation of this fact can only be obtained by complete microsequence analysis of the whole molecule, which would also distinguish the asparagine and glutamine residues from the aspartate and glutamate we have measured in the present work by amino acid analysis.

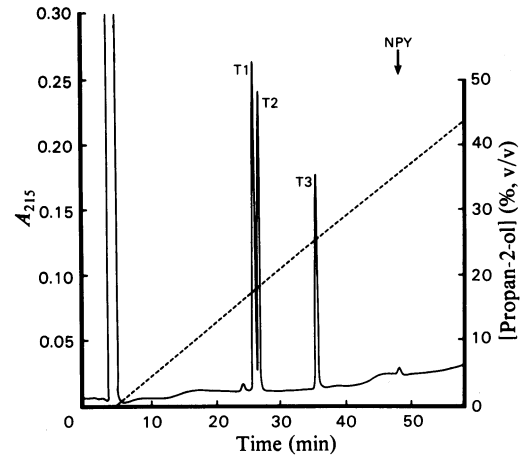

Fig. 6. *Analytical h.p.l.c. of the peptide fragments obtained on tryptic digestion of purified human NPY*

One fraction (17.8–18.3 min) from semi-preparative h.p.l.c. (Fig. 5) was further purified by analytical h.p.l.c. as described in the Materials and methods section. A 2.5 nmol portion of the pure NPY-like peptide was then digested with 1-chloro-4-phenyl-3-tosylamidobutan-2-one-treated trypsin, as described in the Materials and methods section, and the resultant peptide mixture was acidified with 10 μl of trifluoroacetic acid, injected on to a 4.6 mm × 150 mm 5 μm Ultrasphere ODS-silica column eluted with 0.1% trifluoroacetic acid, which after 5 min was developed with a linear gradient of 0–50% propan-2-ol. Fractions were collected and subjected to amino acid analysis. T1, T2 and T3 indicate tryptic fragments representing amino acid residues 1–19, 20–25 and 26–33 respectively of NPY (see the Results and discussion section and Table 2). ——, A_{215}; – – – –, propan-2-ol gradient. The elution position of the purified NPY-like peptide in this system is indicated.

Tryptic cleavage of NPY yields three main peptides T1, T2 and T3 (Fig. 6, and Tatemoto, 1982*b*), and it should also produce the dipeptide Gln-Arg and free tyrosine amide from the *C*-terminus sequence 34–36 (Fig. 1). These were not detected as peaks on h.p.l.c. of the tryptic fragments (Fig. 6), but when the unadsorbed injection peak was subjected to acid hydrolysis and amino acid analysis, glutamate, arginine and tyrosine were all detected. Direct amino acid analysis of this fraction without hydrolysis did not reveal any of these amino acids. However, amino acid analysis of this fraction after treatment with carboxypeptidase B for 90 min showed the release of equimolar amounts of only glutamine and arginine, suggesting that tyrosine was present as free tyrosine amide. This question was resolved by using the system described for tyrosine amide identification with u.v. absorption

Table 2. *Amino acid analyses of the peptides obtained after h.p.l.c. of the tryptic digest of human NPY (Fig. 6)*
Amino acid analysis was performed as described in the Materials and methods section. Compositions are based on the assumption of one arginine residue per tryptic fragment, with the corresponding values for the pig peptide in parentheses. The values shown were obtained by h.p.l.c. separation and detection of fluorescently labelled amino acids, with the exception of proline, which was measured by using ninhydrin detection on the Jeol JLC-6AH automatic amino acid analyser.

	Amino acid composition (mol/mol of peptide)		
	Peptide T1	Peptide T2	Peptide T3
Asp	4.39 (4)	–	0.92 (1)
Glu	2.31 (2)	–	–
Ser	0.85 (1)	0.68 (1)	–
His	–	–	0.73 (1)
Gly	1.04 (1)	–	–
Thr	–	–	1.01 (1)
Arg	1.00 (1)	1.00 (1)	1.00 (1)
Ala	3.00 (3)	1.09 (1)	–
Tyr	0.87 (1)	1.77 (2)	0.72 (1)
Met	0.58 (0)	–	–
Ile	–	–	2.24 (2)
Leu	– (1)	0.82 (1)	1.08 (1)
Lys	1.17 (1)	–	–
Pro	4.14 (4)	–	–

Table 3. *Time-course carboxypeptidase Y digestion of the tryptic fragment T1 (amino acid residues 1-19)*
A sample of peptide T1 (about 0.3 nmol) was incubated at room temperature with 5 μg of carboxypeptidase Y. Samples were taken at the start and after timed intervals; amino acid analysis was performed by h.p.l.c. to identify released amino acids. Arginine was taken to be one residue/molecule for calculation of the release of amino acids; at 95 min maximum predicted arginine release had already occurred and its level did not alter further during the course of the digestion. Except for the amino acids indicated no other amino acids were detected during the 365 min period of the investigation.

	Released amino acids (mol/mol of peptide)			
	95 min	160 min	275 min	365 min
Arg	1.00	1.00	1.00	1.00
Ala	0.90	0.91	0.93	0.95
Met	0.78	0.91	0.91	1.02
Asp	0.29	0.41	0.70	0.90
Glu	0.12	0.17	0.23	0.45

at 280 nm. Tyrosine standard was unretained, being eluted within 5 min of injection at virtually 0% acetonitrile, whereas tyrosine amide was adsorbed on the column and was eluted after 22 min at approx. 10% acetonitrile. No free tyrosine peak was measured in the sample of unadsorbed injection material obtained on h.p.l.c. of the tryptic fragments, concurrent with the data obtained by direct amino acid analysis without hydrolysis, but a u.v.-absorbing peak was obtained by this method with the elution characteristics of tyrosine amide.

NPY was first isolated and characterized from pig brain (Tatemoto, 1982*b*), and immunohistochemical studies have demonstrated the presence of NPY-like material in peripheral noradrenergic neurons (Lundberg *et al.*, 1982). It has also been suggested that APP-like immunoreactivity in catecholaminergic systems, including the adrenal medulla (Lundberg *et al.*, 1980), is due to cross-reactivity with the structurally related NPY (Lundberg *et al.*, 1982). The purification and chemical characterization of a NPY-like peptide from human adrenal-medullary tumours provides further evidence to support the idea that NPY-like immunoreactivity in the peripheral sympathetic nervous system is due to the presence of the brain peptide NPY.

Human adrenal-medullary NPY appears to only differ from the pig peptide in the replacement of leucine at position 17 by a methionine residue; this is not unusual, since it can be explained at the genetic level by a single base alteration. Interestingly human (Fig. 1), pig, sheep, bovine and dog pancreatic polypeptides (reviewed by Lovonics *et al.*, 1981) all have methionine at position 17, whereas pig PYY (Tatemoto, 1982*a*) and NPY (Tatemoto, 1982*b*; Fig. 1) and avian pancreatic polypeptide (Kimmel *et al.*, 1975; Fig. 1) have leucine. Thus the presence of methionine in human NPY supports the suggestion that these peptides are a biosynthetically or genetically related family of peptides (Tatemoto, 1982*b*).

Pig NPY has been shown to have potent vasoconstrictor properties (Lundberg *et al.*, 1982), hence the possibility of adrenal medullary release of NPY during stress has many implications. Furthermore patients with adrenal-medullary tumours are recognized by their raised circulating catecholamine concentrations and hypertensive symptoms, but the biosynthesis and possible secretion of NPY by these tumours may also contribute to some of the cardiovascular changes that have been reported (Manger & Gifford, 1977).

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