STUDIES ON THE PRIMARY STRUCTURE AND BIOLOGICAL ACTIVITY OF A HUMAN NEUROPHYSIN

Irene FOSS, Knut SLETTEN and Olav TRYGSTAD
Section of Endocrinology, Institute of Pediatric Research, Rikshospitalet, Oslo 1, Norway

and

Department of Biochemistry, University of Oslo, Oslo 3, Norway

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1. Introduction

Several novel pituitary polypeptides have been found to have potent lipolytic activity [1]. From a heterogeneous human pituitary lipid-mobilizing fraction we have previously isolated a highly active lipolytic polypeptide [2]. The fraction also contained a larger protein with lipolytic activity, and the aim of this work was to purify and characterize that protein. Our results indicate that it belongs to the so called neurophysins; which are located in the neurohypophysis [3], and form reversible complexes *in vitro* with oxytocin and vasopressin [4, 5]. The neurophysins are acidic proteins with molecular weights in the range of 10,000 [4, 5]. They possess characteristic UV absorption spectra with no maximum around 280 nm [5]. Recently human neurophysin was prepared from acetone-dried pituitary powder [6]. So far studies on the primary structure or on biological activities of human neurophysin have not been published.

We introduce here a new method for preparation of human neurophysin from fresh frozen pituitary glands. The protein consists of 87 amino acid residues. The sequence of the first 16 amino acids as well as the COOH-terminal residues are presented. The protein binds reversibly oxytocin and lysine-vasopressin. It exhibits lipolytic and hyperglycemic effects as well as a lowering effect on serum amino acids in rabbits. It induces lipolysis *in vitro* in rabbit and human adipose tissue, but has no lipolytic effect in mouse fat pads.

2. Materials and methods

All chemicals were reagent grade, and those used for Edman degradation were further purified [7].

Lysine-vasopressin was generously supplied by Sandoz (Basel), and oxytocin, Type II, and carboxypeptidase A were purchased (Sigma Chemical Co., St. Louis).

Frozen human pituitary glands were homogenized and extracted at pH 8.5. The lipid-mobilizing fraction was obtained by precipitation with acetone according to the method described previously [2]. All steps in the preparation procedure were performed at 0–4°.

The Sephadex gel filtrations, and the DEAE-cellulose (Eastman Rochester) anion exchange chromatography were performed as described earlier [2]. Binding of the protein with lysine-vasopressin and oxytocin was examined by gel filtration on Sephadex G-50 columns equilibrated with 0.1 M phosphate buffers with pH from 5.4 to 8.5.

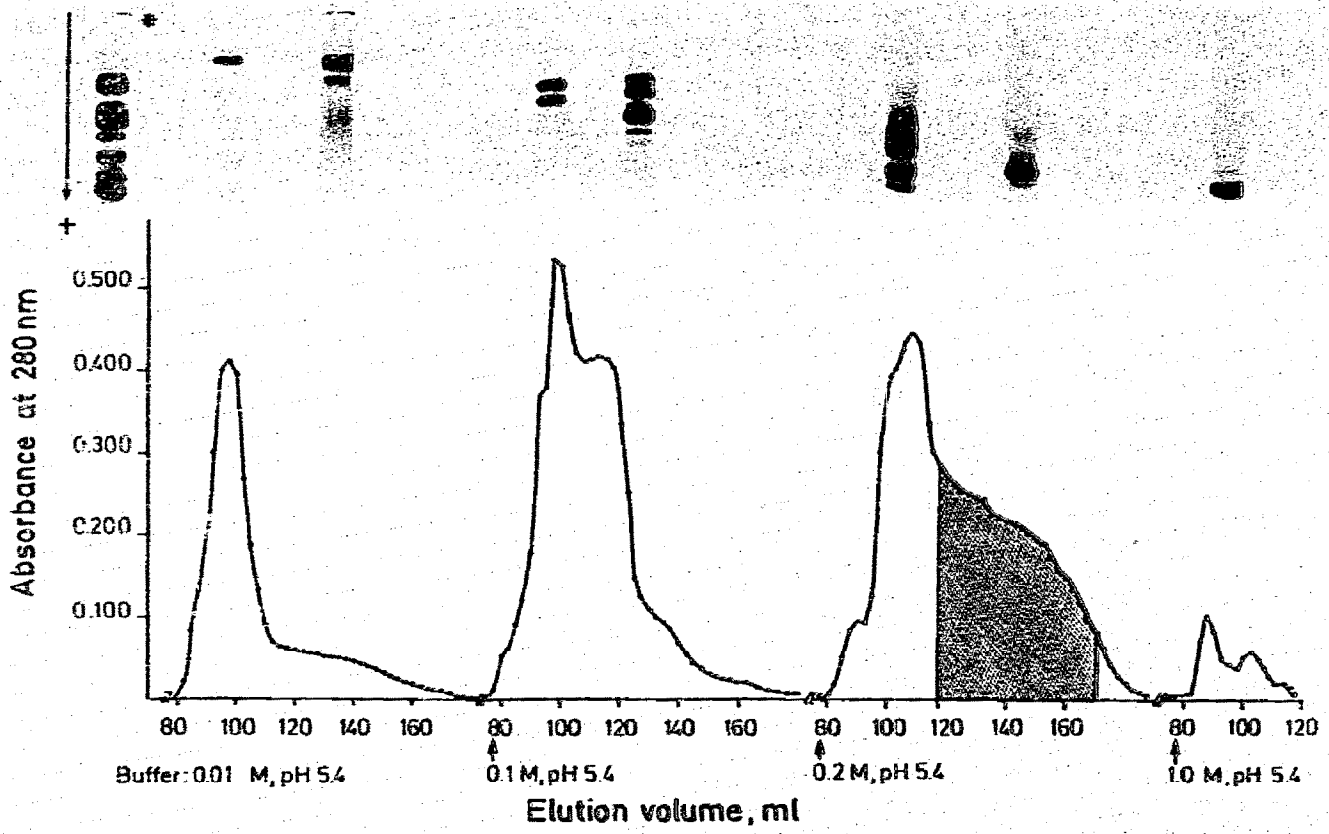

Fig. 1. Chromatography of the lipid-mobilizing fraction prepared from 200 g fresh frozen human pituitary glands on a column (2.4 × 30 cm) of DEAE-cellulose equilibrated with 0.01 M phosphate buffer pH 5.4. The column was eluted at constant pH by stepwise increase of molarity, and a flow rate of 200 ml/hr. The shaded area indicates fractions used for Sephadex gel filtration. The insets show the electrophoretic patterns of the protein applied to the column*, and of the proteins eluted. Approx. 0.2 mg samples of lyophilized materials were run at pH 8.7 on 7.5% polyacrylamide gel columns.

The protein contents in the effluents were determined spectrophotometrically at 280 nm or by a ninhydrin method [8].

Disc electrophoresis was carried out on 7.5% polyacrylamide gel columns at pH 8.7 [cf. 2].

Amino acid analysis and Edman degradation were carried out as described earlier [7]. Carboxyl-terminal analysis was performed on oxidized protein [7] with carboxypeptidase A in 0.1 M NH_4HCO_3, pH 8.4, at 37° with an enzyme:substrate ratio of 1:20 on a molar basis. The samples were lyophilized and studied in the amino acid analyser.

Non-esterified fatty acids (NEFA) were quantified by a modified Dole's titration procedure, and blood glucose by an o'Toluidin method [cf. 2]. Serum glycerol was determined according to "Biochemica Test Combination" (Boehringer, Mannheim).

In vitro lipolysis was studied in human subcutaneous fat pads obtained during laparotomy from three fasting females operated under barbital anesthesia because of uterine myoma, and in peritoneal fat pads obtained from the albino mouse and rabbit [cf. 2].

In vivo studies of lipolysis and changes in blood glucose concentrations were observed in albino rabbits of either sex with body weights of more than 4 kg. Blood samples for determinations of serum NEFA, glycerol, and blood glucose were taken before the injection of 0.1 mg protein, and 1, 7, and 24 hr after the injection.

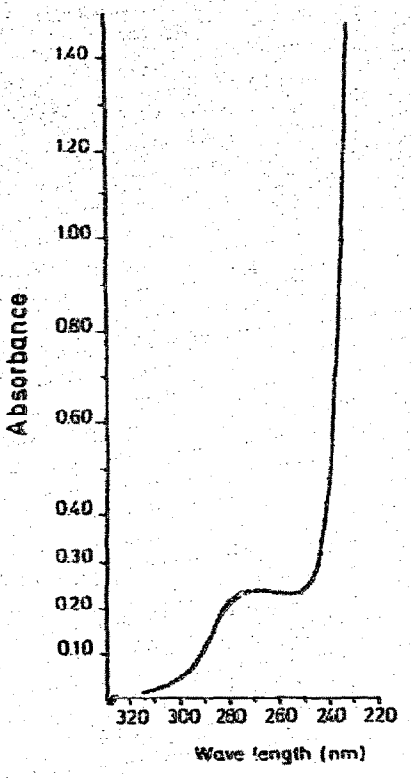

Fig. 2. Ultraviolet absorption spectrum of the human pituitary protein from the shaded area fig. 1. The concentration of the sample was 0.4 mg per ml in 0.1 M phosphate buffer pH 7.0.

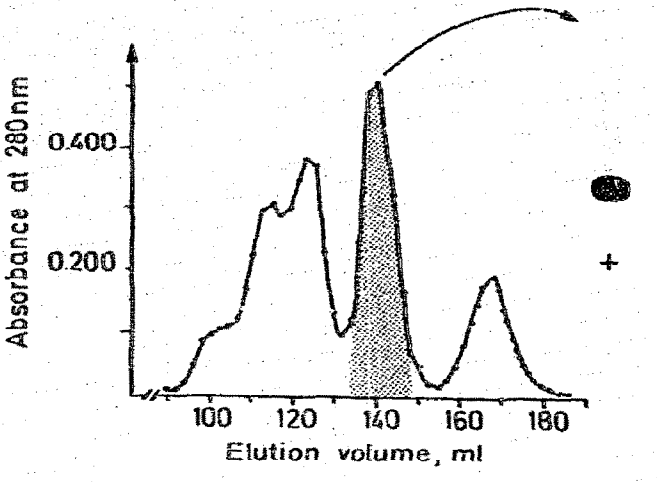

Fig. 3. Filtration on a Sephadex G-50 gel column (1.6 × 110 cm) equilibrated with 0.1 M phosphate buffer pH 7.5 of 30 mg protein obtained from the DEAE-cellulose chromatography of the human pituitary lipid-mobilizing fraction (the shaded area fig. 1). The eluant with the characteristic absorption spectrum (cf. fig. 2) is indicated by the shaded area. The inset shows the electrophoretical pattern of 0.2 mg of this material applied to a 7.5% polyacrylamide gel column run at pH 8.7.

3. Results and discussion

The lyophilized human pituitary lipid-mobilizing fraction obtained by Sephadex G-100 gel filtration [2] was dissolved in 0.01 M phosphate buffer pH 5.4, and applied to a DEAE-cellulose column (2.4 × 30 cm). The elution was carried out by stepwise increase in the ionic strength of the employed phosphate buffer. Fig. 1 depicts the distribution of the protein fractions and their electrophoretical patterns on polyacrylamide gel. The lipid-mobilizing agent prepared previously [2] appears in the starting buffer. Several of the more acidic molecules are also biologically active, and studies on some of these polypeptides are in progress. The 0.2 M buffer eluted a lipolytic polypeptide where the usual protein peak at 280 nm in the absorption spectrum leveled out to a horizontal line (fig. 2). In order to achieve maximum purification of this polypeptide only material recovered from the descending part of the peak was collected for further purification by gel filtration on Sephadex G-50 columns (1.6 × 110 cm) equilibrated with 0.1 M phosphate buffer pH 7.5. There appeared four protein peaks, fig. 3, and the material in the third peak showed the characteristic UV absorption spectrum. The main fractions were used in a subsequent refiltration on the same column, and the protein was eluted as a symmetrical peak. The elution volume corresponded to a molecular weight of about 10,000 [2]. The yield of the purified protein was approx. 0.05 mg/g of wet pituitaries. The absorbance at 280 nm was 1.00 for 1.6 mg of the protein dissolved in phosphate buffer pH 7.0.

The protein moved as a single protein band in polyacrylamide gel electrophoresis, and the NH_2-terminal sequence analyses resulted in one residue, only, which indicates that the protein is at least 95% pure. The results from the amino acid analyses of the protein are listed in table 1. The amino acid composition is based upon a molecular weight of about 10,000 and 7 residues of aspartic acid. The minimum molecular weight was calculated to be 8983. A small content of methionine and tryptophan suggests a

Table 1
Amino acid composition of human neurophysin.

Amino acid	Number of residues found*	
Aspartic acid	7.09	7
Threonine	1.65	2
Serine	4.08	4
Glutamic acid	10.2	10
Proline	7.21	7
Glycine	12.1	12
Alanine	9.28	9
Half-cystine	12.9	13
Valine	2.85	3
Methionine	0.34	0
Isoleucine	0.98	1
Leucine	6.78	7
Tyrosine	1.08	1
Phenylalanine	2.56	3
Histidine	0.93	1
Lysine	3.36	3
Arginine	3.98	4
Tryptophan	0.25	0
Molecular weight 8,983		

* Mean values from 5 different analyses after 24 hr hydrolysis

** Determined as cysteic acid after performic acid oxidation

The residue numbers are based upon 7 residues of aspartic acid

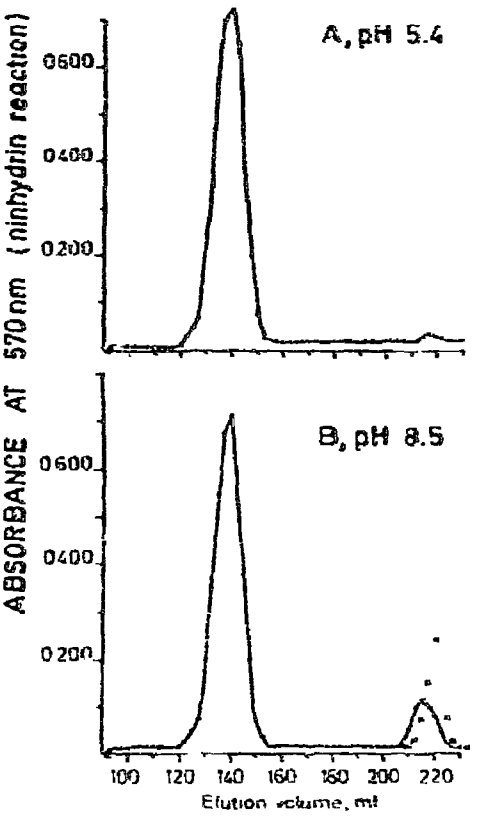

Fig. 4. Repeated gel filtrations on Sephadex G-50 columns (1.6 × 110 cm) of a mixture consisting of 10 mg purified human pituitary protein (shaded area fig. 3) and 1 mg oxytocin or 1 mg lysine-vasopressin in 0.1 M phosphate buffer. A) The elution pattern at pH 5.4. B) The elution pattern at pH 8.5 (in the second peak the solid line indicates oxytocin, and the broken line lysine vasopressin). Peptide material was determined by the ninhydrin reaction [8].

microheterogeneity, possibly due to the presence of a second neurophysin.

The UV absorption spectrum [5], the molecular weight [4, 5], and the high content of half cystine and glutamic acid [1] are characteristic for the neurophysins. Therefore the protein was tested for binding of lysine-vasopressin and oxytocin [6]. Fig. 4A shows the result from gel-filtration of 10 mg of the protein with 1 mg of lysine vasopressin or oxytocin at pH 5.4 and fig. 4B shows the elution pattern of the mixture at pH 8.5. At pH 5.4 to pH 7.0 only one polypeptide peak was revealed, whereas 2 peaks appeared at higher pH. From these data it is evident that the protein binds the two neurohypophyseal hormones at a pH below 7.0. Consequently, this purified human pituitary protein belongs to the neurophysins.

The amino acid composition of our protein differs from that which Cheng and Friesen prepared from acetone dried human pituitary powder [6], particularly in the content of arginine, proline, glycine, and alanine. So far sequence studies have been published for bovine neurophysin -I and -II [9, 10] and porcine neurophysin I [11], only. In fig. 5 the 18 NH_2-terminal residues of these neurophysins are compared with those determined for our human neurophysin. The sequence is most similar to bovine neurophysin-I, and differs only in positions 2, 3, and 9, which can all be accounted for by one single base change in the genetic code.

Incubation with carboxypeptidase A yielded the following COOH-terminal sequence. (Thr, Val, Phe, Ala)—Leu-COOH, that of porcine neurophysin-I was leucine/phenylalanine [11].

In vitro lipolysis occurred in rabbit as well as in human fat pads after addition of human neurophysin

	1	5	10	15
Human neurophysin	Ala–Ala–Pro–Asp–Leu–Asp–Val–Arg–Lys–Cys–Leu–Pro–Cys–Gly–Pro–Gly–(Asx, Lys) –			
Bovine neurophysin I [9]	Ala–Val–Leu–Asp–Leu–Asp–Val–Arg–Gln–Cys–Leu–Pro–Cys–Gly–Pro–Gly–Gly–Lys–			
Bovine neurophysin II [10] / Porcine neurophysin I [11]	Ala–Met–Ser–Asp–Leu–Glu–Leu–Arg–Gln–Cys–Leu–Pro–Cys–Gly–Pro–Gly–Gly–Lys –			

Fig. 5. The NH_2-terminal amino acid sequences of neurophysins.

(table 2). The minimal effective concentration was 0.1 μg/ml, one tenth of that for vasopressin, demonstrating that the lipolysis could not be explained by a contamination with vasopressin. Oxytocin showed no lipolytic effect in human or rabbit fat pads. In contrast in mouse fat pads lipolysis occurred in the presence of oxytocin and vasopressin, but not with neurophysin. Furthermore, our alkaline extraction procedure should have dissociated oxytocin and vasopressin from neurophysin.

A significant *in vivo* lipolytic effect of human neurophysin was observed in the rabbit (fig. 6). Their serum NEFA increased from 0.26 to 2.76 meq/ℓ 1 hr after the subcutaneous injection of neurophysin, and there was a similar increase of serum glycerol. This agrees with the increase of serum NEFA following injection of neurophysin in monkeys, who showed an increase of blood glucose as well [1]. Also in the rabbit the blood glucose concentration rose to a maximum after 24 hr (table 3). The serum calcium concentration (data not given) showed no change following the injection of human neurophysin [cf. 2]. Furthermore, in rabbits and monkeys neurophysin showed an unexpected effect on protein metabolism with a decrease of serum amino acids [1]. A similar effect has been observed in rabbits with our protein (to be published). The metabolic effects described are similar to those attributed to growth hormone, but the initial lipid-mobilizing fraction here used had no somatotropin contamination [2].

Table 2

In vitro release of non-esterified fatty acids (NEFA) from human, rabbit, and mouse fat pads after addition of human neurophysin, vasopressin, or oxytocin.

Preparation added (μg/1.1 ml)	Mean release of NEFA (μmole/100 mg · 3 hr ±SE) Human fat	Rabbit fat	Mouse fat
Fat pads blanks (no addition)	0.29 ± 0.03	0.31 ± 0.02	0.30 ± 0.02
Human neurophysin			
10	1.35 ± 0.07		0.29 ± 0.01
1	0.81 ± 0.02	1.19 ± 0.05	
0.1	0.50 ± 0.03	0.70 ± 0.07	
0.01	0.35 ± 0.03	0.36 ± 0.05	
Lysine-vasopressin			
10	0.81 ± 0.03	0.56 ± 0.05	0.81 ± 0.04
1	0.47 ± 0.03	0.35 ± 0.04	
0.1	0.32 ± 0.02		
Oxytocin			
10	0.30 ± 0.03	0.35 ± 0.02	0.62 ± 0.06
1	0.27 ± 0.04	0.29 ± 0.03	

All assays were run in quadruplicate.

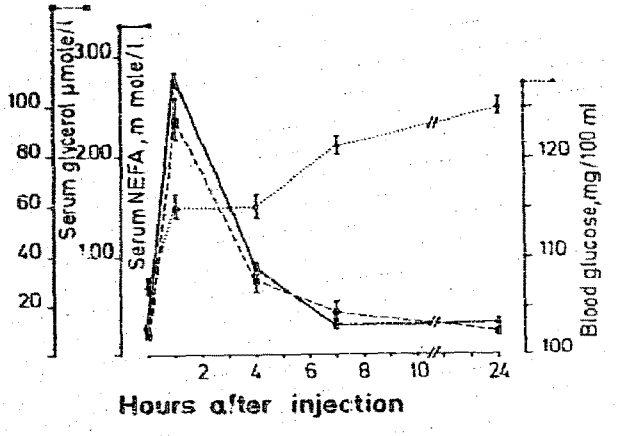

Fig. 6. *In vivo* effect of subcutaneous injection of 0.1 mg human neurophysin on serum non-esterified fatty acids (NEFA, ——), glycerol (– – –), and blood glucose (------) in rabbits. The curves show the mean concentrations after injection into 10 rabbits, the vertical bars indicate the standard errors of the means.

Acknowledgements

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