A COMPARATIVE STUDY OF THE LIPID CONTENT AND LECITHIN-BINDING CAPACITY OF BOVINE NEUROPHYSINS

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1. Introduction

One possible way to assess the biological function of neurophysin is to analyze in vitro the formation of heterologous complexes between the so-called carrier-protein and its hormonal ligands, ocytocin and vasopressin [1–3]. Recent reports from this laboratory have contributed to clarify some aspects of the binding process [4–6] and to demonstrate the allosteric properties and half-sites reactivity of the system [6]. Since most of the binding studies were performed on protein samples extracted from acetone-dried pituitaries [7], it was objected, by some authors [8], that they might deal with non-native protein, artefactually delipidated. These authors also questioned the fact that both the oligomerising and the binding capacities of the protein might be different when presumably associated to lipids compared with the situation where the lipids will be dissiociated from the protein during the preparation.

In view of the known high lipid content of the neurosecretory granules membranes of the neurohypophysis neurons [9], one possibility was that those proteins might exist as acidic lipoproteins inside the granules [8,10]. Because of the biological importance of such questions, we have investigated systematically this possibility. Careful comparative analysis of the lipid content of the proteins extracted under various mild versus drastic conditions, in connection with experiments of reconstitution in vitro, show that neurophysins associate weakly only a discrete number of lecithin molecules, thus rendering unlikely their classification as soluble lipoproteins.

Abbreviations: DPPC, dipalmitoyl phosphatidyl choline; NI, neurophysin I; NII, neurphysin II.

2. Materials and methods

The various crude bovine neurophysin preparations are described in table 1. Bovine neurosecretory granules were obtained according to the procedure of Dean and Hope [11]. NI and NII were purified by isoelectric focusing as previously described [1].

Lecithin preparations were obtained by dispersion of [^{14}C]phosphatidylcholine (1.765 Ci/mmole, NEN) and DPPC (A grade, Calbiochem) at 48–50°C (DPPC transition temperature = 41°C) [12] for 30 min in 10 mM phosphate buffer containing 1 mM EDTA pH 7.4, then sonication with a Bronwill biosonicator at 0–5°C and in pure nitrogen atmosphere. They were used immediately and absence of lysolecithin was checked [13].

NI or NII were allowed to react with lecithin preparation by incubation at 42°C for 2 h then the mixture was analyzed by ultracentrifugal flottation in linear density gradients of KBr (d 1.06 to 1.24) or sucrose (d 1.04 to 1.19) in 10 mM phosphate buffer, 1 mM EDTA, pH 7.4, prepared with a Buchler gradient maker. Ultracentrifugation was carried out at 5°C in a Beckman L2 65 B Ultracentrifuge using a SW 65 Ti rotor at 60 000 rev/min for 64 h (KBr) or 81 h (sucrose). The density of each fraction was measured by the refractive index on an Abbe refractometer. Protein content was determined by the Lowry procedure [14]. Radioactivity was measured using a Intertechnique SL 30 liquid scintillation

Table 1
Fatty acid contents of various crude bovine neurophysin preparations

Starting material	Extraction	Precipitation	Gel filtration	Percentage of total fatty acids in neurophysin	Lecithin/ neurophysin molar ratio
A Acetonic powder	0.1 N HCl	NaCl	Sephadex G-25 Sephadex G-75	0.4	<0.1
B Fresh glands	0.1 N HCl	NaCl	Sephadex G-25 Sephadex G-75	0.5	0.1
C Fresh glands	0.1 N HCl Triton X-100	NaCl	Sephadex G-25: Triton X-100 Sephadex G-75	1.5	0.25
D Fresh glands	20 mM Phosphate buffer pH 7	0	Sephadex G-75 20 mM phosphate buffer pH 7	2.8	0.5
E Neurosecretory granules	5 mM Phosphate buffer pH 7	0	0	4	0.7

(A) (B) (C) The protein–hormone complex was prepared according to Hollenberg and Hope [7] then neurophysins were separated by Sephadex G-25 and G-75 filtrations (in 0.1 N HCOOH). (D) Fresh glands extracted at neutral pH. Neurophysins were separated by Sephadex G-75 filtration (pH 7). (E) Neurophysins extracted by lysis of the neurosecretory granules at neutral pH at 5°C overnight. The insoluble material was eliminated by centrifugation at 101 000 *g* for 60 min at 5°C. Percentage of total fatty acids expressed in μg of palmitic and stearic acids per 100 μg of protein.

counter. Phosphorus determination was made after perchloric digestion of the samples [15].

Analytical thin layer gel isoelectric focusing was performed in the LKB multiphor using a 5% polyacrylamide gel containing 2% LKB ampholine pH 4–6 and 13% sucrose at 5°C. The pH gradient was measured with a surface pH electrode and protein bands were revealed by 10% trichloracetic acid as white strips.

The fatty acid content of the neurophysin preparation was estimated by gas-liquid chromatography of the methyl esters [16] using a Varian Aerograph model 2100. Quantitative estimation was obtained with a C_{17} fatty acid internal standard. Circular dichroism (CD) spectra were recorded on a Jouan model III CD dichrograph. Path length was 0.1 cm. Spectra were recalculated as molar ellipticities (deg. cm^2/dmole) using mol. wt. of 9560 (NI) and 10 040 (NII). Equilibrium dialysis was carried out as previously described [1].

3. Results

Various neurophysin preparations were analyzed with respect to their fatty acid content (table 1) and their isoelectric focusing behavior (fig.1). The fatty acids detected are palmitic and stearic acids with ratio equal to 2 : 1 except for E (1 : 1). The percentage never exceeds 4%. From the total fatty acid percentages, it is possible to evaluate the lipid-neurophysin molar ratio of each preparation (table 1) assuming that the major part of the lipid content is constituted by diacyl phosphoglycerides. This hypothesis is supported by the fact that the phosphorus concentration measured on crude neurophysins extracted by 0.1 N HCl corresponds to the one calculated from total fatty acids percentage. Thus, crude neurophysins obviously contain a low proportion of lipid since the molar ratio never exceeds 0.7. As expected, the low content of lipid did not modify the isoelectric focusing (fig.1) behavior of the neurophysins. From these observations, it can be concluded that the interactions between neurophysin and granular lipids must be weak seeming not to resist the mildest extraction conditions.

Analysis in a KBr linear density gradient of the neurophysin-DPPC interactions showed that

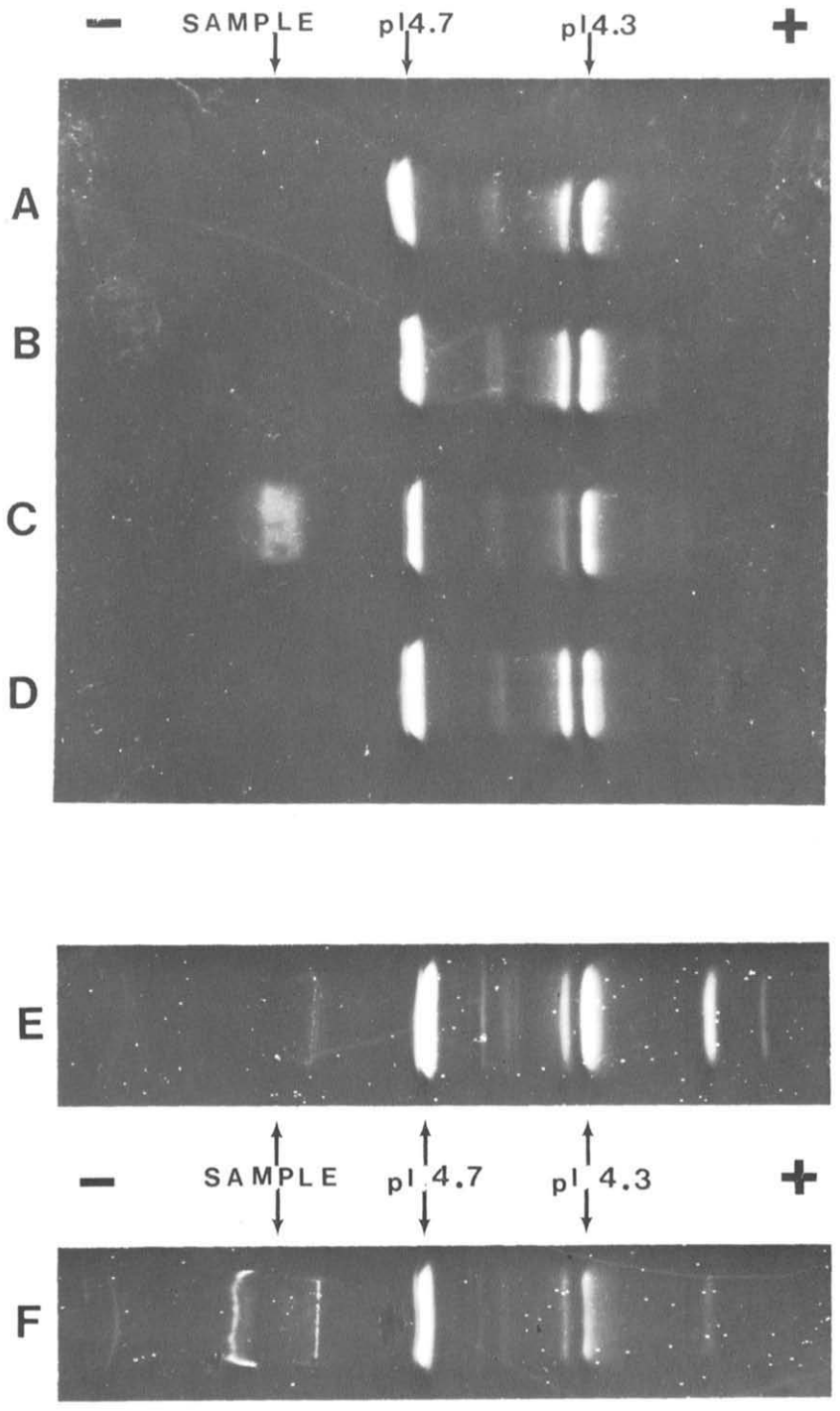

Fig. 1. Thin layer gel isoelectric focusing of crude bovine neurophysin preparations in a pH gradient (4 to 6). The various protein samples (~ 100 μg) A, B, C, D, E correspond to the respective A, B, C, D, E crude bovine neurophysin preparations (table 1). Sample F was obtained from neurosecretory granules disrupted in the presence of 0.2% Triton X-100.

neurophysin behavior was the same in the presence or in the absence of phospholipids (fig.2). With 0.07 mM NII, a weak proportion of the radioactivity was detected in the fractions containing neurophysin (representing approx. 0 to 2 mol of lecithin per mol of neurophysin). Therefore, a very weak interaction is only detected at low protein concentration where the monomeric form of neurophysin is predominant [6].

In a sucrose linear density gradient (fig.3), the major part of the NII incubated with DPPC had the same behavior as the protein alone but approx. 5%

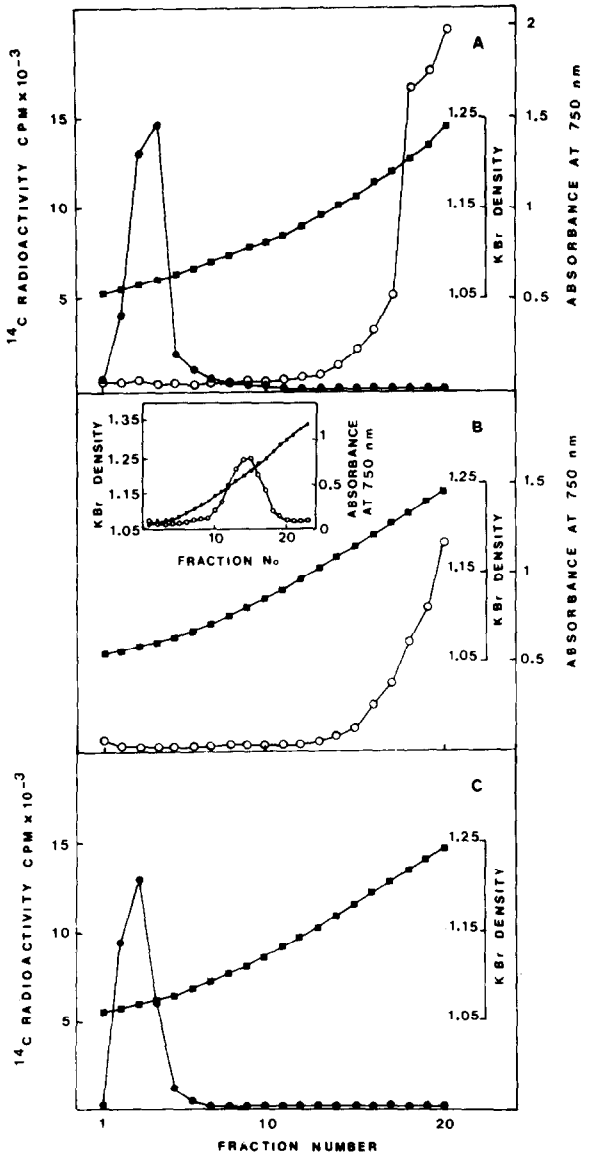

Fig.2. Isopycnic centrifugation in a KBr gradient. (A) 0.3 ml of 10 mM DPPC–0.4 mM N I; (B) 0.3 ml of 0.33 mM N I; (C) 0.3 ml of 10 mM DPPC. Fraction volume = 0.25 ml. Protein (Lowry determination): absorbance at 750 nm (open circles). Lecithin: radioactivity of 20 μl aliquots (closed circles). Density (squares).

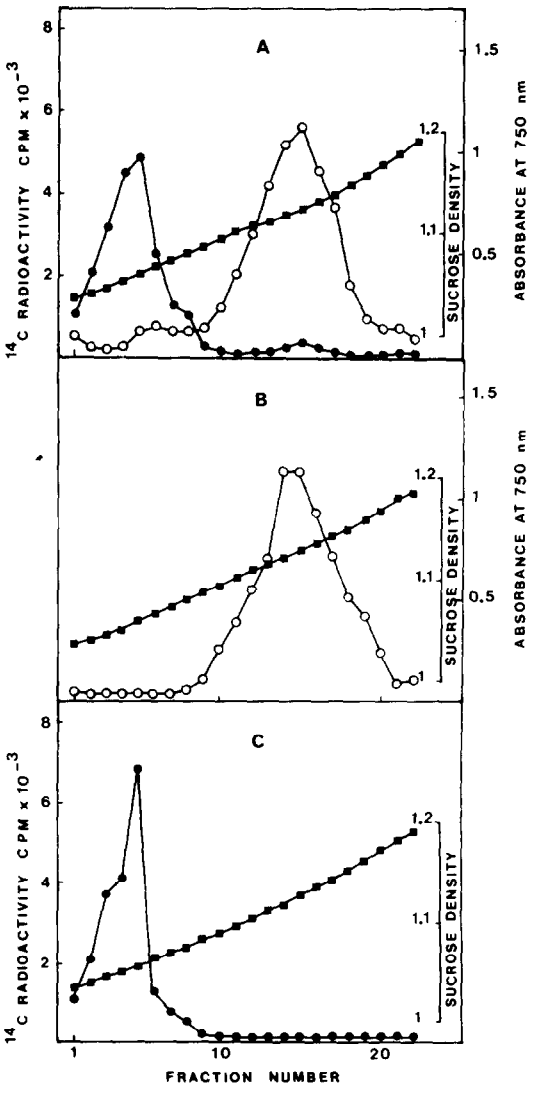

Fig.3. Isopycnic centrifugation in a sucrose gradient. A, 0.3 ml of 11 mM DPPC–0.33 mM N II; B, 0.3 ml of 0.33 mM N II; C, 0.3 ml of 11 mM DPPC. Fraction volume = 0.23 ml. Symbols as in fig.2 legend.

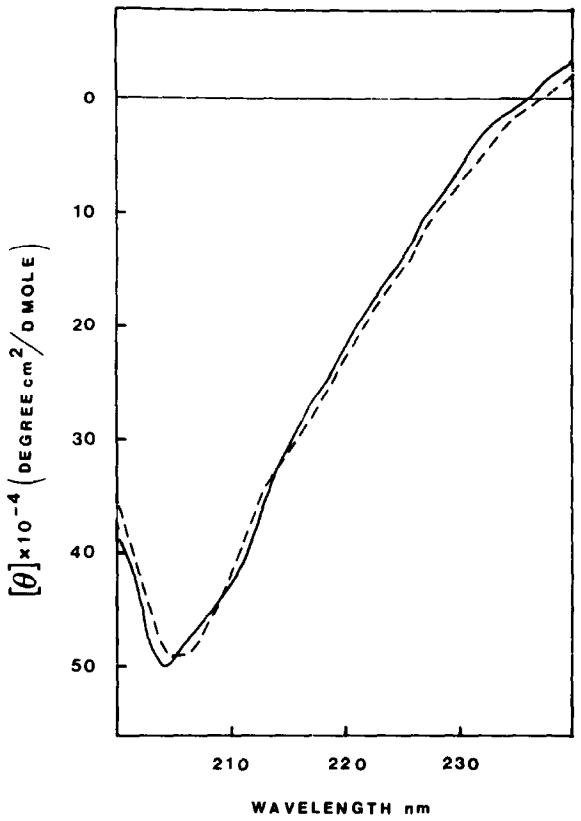

Fig.4. Far u.v. CD spectra of 20 μM N I alone (solid line) and incubated with 1 mM DPPC at 42°C for 2 h (broken line) at pH 7.4. The contribution of DPPC at each wavelength was substracted from the spectrum of NI–DPPC mixture. The standard deviation at all wavelength never exceeded 10% of the molar ellipticity.

were detected at d 1.073 and 3 per cent of the lipids were recovered at d 1.124. This corresponds to a DPPC : neurophysin ratio equal to 1. If the protein concentration decreased in a NII-DPPC mixture, the radioactivity at d 1.124 increased. The ratio was approximately equal to 1.5 and 2 with 0.18 mM and 0.07 mM NII. Here again, a slightly greater interaction was detected when protein concentrations favored the monomeric neurophysin [6]. In a barbital buffer, the NII : DPPC distribution patterns were similar in the presence of Ca^{2+} or in its absence.

Fig.4 shows the CD spectra of NI alone and in the presence of a 50-fold molar excess of DPPC. No significant difference was observed. Addition of lecithin to NI seems to induce neither an increase in the α-helical content of the protein nor a measurable modification of its tertiary structure. The same result was obtained with NII.

In order to examine the hypothesis of an acidic lipoprotein form of the neurophysins with a high affinity for the neurohypophyseal hormones [8], we studied the effect of added DPPC on the binding of ocytocin to NII. The isotherm is similar to that obtained in the absence of lecithin (fig.5). The number of sites ($n \sim 1$) and the apparent association constant ($K_A \sim 1{\cdot}10^5$ M^{-1}) are not significantly different from these determined with neurphysin alone ($n = 1, K_A = 2.5{\cdot}10^5$ M^{-1}) under similar concentration conditions [6]. The apparent decrease of K_A from 2.5 to $1{\cdot}10^5$ M^{-1} is well within the experimental variations usually observed when this constant is approximated by a direct extrapolation of the highest slope of the curve.

4. Discussion

Great interest arose from the recent reports [10,8] that neurophysins in their 'native' state might be

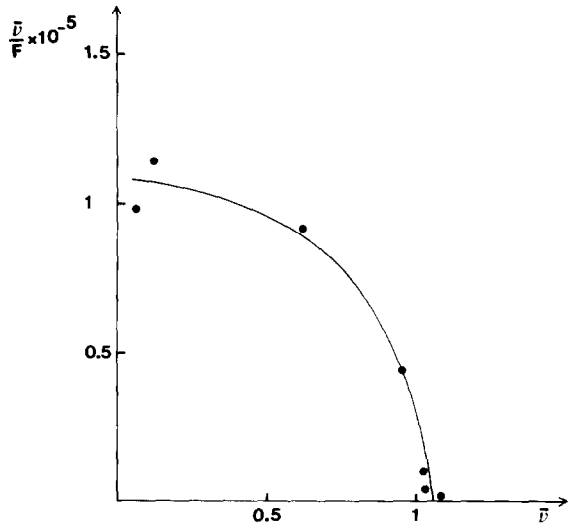

Fig.5. Scatchard plot of the binding of ocytocin (from 4 to 750 μM) in 0.1 M acetate buffer pH 5.6 at 24°C to NII (50 μM) in the presence of 0.25 mM DPPC. $\bar{\nu}$ = bound ocytocin concentration per molar concentration of protein and F is the free ocytocin concentration measured at equilibrium.

associated to lipids, thus displaying different chemical and physical properties from the delipidated proteins. We have carefully analyzed such a hypothesis by both direct and indirect approaches. From the direct observation of the physicochemical properties of neurophysins extracted under various conditions, it appears that they do not differ from each other significantly. This suggests that neurophysin obtained by the classical procedure from acetone-dried glands is probably convenient for binding studies.

From the reconstitution experiments, CD spectra and binding measurements, it can be concluded that purified neurophysins do not interact strongly with lecithins, one of the major constituents of the neurosecretory granule membranes. However, the protein monomer had a slightly higher capacity for lipid binding than the dimeric form suggesting that the contact areas involved in this weak interaction might be common to the hydrophobic area of the dimerization reaction.

In conclusion, these observations make the hypothesis that 'native-neurophysins' are soluble lipoproteins rather unlikely. Nevertheless, the possibility that neurophysin or neurophysin–hormones complexes might possess some neurosecretory granules membrane binding sites of still unknown nature cannot be excluded at this time.

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References

[1] Camier, M., Alazard, R., Cohen, P., Pradelles, P., Morgat, J. L. and Fromageot, P. (1973) Eur. J. Biochem. 32, 207–214.
[2] Cohen, P., Camier, M., Wolff, J., Alazard, R., Cohen, J. S. and Griffin, J. H. (1975) Ann. N.Y. Acad. Sci. 248, 463–479.
[3] Breslow, E. (1975) Ann. N.Y. Acad. Sci. 248, 423–441.
[4] Griffin, J. H., Alazard, R. and Cohen, P. (1973) J. Biol. Chem. 248, 7975–7978.
[5] Wolff, J., Alazard, R., Camier, M., Griffin, J. H. and Cohen, P. (1975) J. Biol. Chem. 250, 5215–5220.
[6] Nicolas, P., Camier, M., Dessen, P. and Cohen, P. (1976) J. Biol. Chem. 251, in press.
[7] Hollenberg, M. D. and Hope, D. B. (1968) Biochem. J. 106, 557–564.
[8] Pliska, V. and Meyer-Grass, M. (1975) Ann. N.Y. Acad. Sci. 248, 235–245.
[9] Vilhardt, H. and Holmer, G. (1972) Acta Endocrinol. 71, 638–648.
[10] Mylroie, R. and Koenig, H. (1971) J. Histochem. Cytochem. 19, 738–746.
[11] Dean, C. R. and Hope, D. B. (1967) Biochem. J. 104, 1082–1088.
[12] Hauser, H., Henry, R., Leslie, R. B. and Stubbs, J. M. (1974) Eur. J. Biochem. 48, 583–594.
[13] Skipski, V. P., Peterson, R. F. and Barclay, M. (1964) Biochem. J. 90, 374–377.
[14] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[15] Fiske, G. H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400.
[16] Ben Abdelkader, A., Cherif, A., Demandre, C. and Mazliak, P. (1973) Eur. J. Biochem. 32, 155–165.