BIOCHIMIE, 1974, 56, 61-76.

Conformational studies on muscular parvalbumins.

II. Nuclear magnetic resonance analysis (*).

Joseph PARELLO, Adrien CAVÉ, Pedro PUIGDOMENECH (**), Catherine MAURY (***), Jean-Paul CAPONY and Jean-François PECHÈRE.

Equipe de Recherche de Biophysique n° 140 (CNRS), Université des Sciences et Techniques du Languedoc, 34000 Montpellier, France
Département de Biochimie Macromoléculaire du CNRS, Route de Mende, B.P. 5051, 34033 Montpellier — Cedex, France.

(5-6-1973).

Summary. — NMR spectroscopy (proton resonance at 100 and 270 MHz) has been used to observe conformational features in muscular parvalbumins from hake (*Merluccius merluccius*) and carp (*Cyprinus carpio*), in the native state, in the denatured state (6M guanidinium chloride or heating) and after almost complete removal of calcium ions. From these observations it appears that the removal of the strongly bound calcium ions leads to a structure very similar to that obtained by chemical or thermal denaturation. On the other hand, the NMR spectrum of the native carp parvalbumins can be interpreted in the light of the recent X-Ray data obtained elsewhere for this protein. Some aspects of the primary structure of these parvalbumins, such as the presence of an N-terminal acetyl residue, have also been investigated with the NMR technique.

INTRODUCTION.

Previous results of optical measurements [1, 2] have indicated the presence in several muscular parvalbumins from lower vertebrates of a high degree of order (*ca* 40 p. cent helicity), a situation which is amply documented in the recent X-Ray analysis of the crystals of one of the components (p*I* 4.25) from carp muscle [3, 4]. The latter studies have also confirmed the globular character of these proteins which was hitherto inferred from hydrodynamic studies [5] and they have provided a detailed description of their tertiary structure and, in particular, of the configuration of the Phe residues whose very high proportion make up for one of the most remarkable features of muscular parvalbumins [6]. As part of a series of physicochemical experiments directed towards the knowledge of their conformation, the NMR analysis of this class of proteins from lower vertebrates therefore appears to be very promising and the more so that it is made easy by their excellent solubility properties. The interest of such an analysis further derives from the recent finding that parvalbumins strongly bind 2 Ca^{++}/mole [7, 8], a situation which points towards a possible functional analogy of these proteins with the muscle regulatory protein troponin-C, found in higher vertebrate muscles. NMR spectra indeed should be very helpful in the detection and identification of conformational changes associated with the binding and release of calcium ions by parvalbumins and troponin. Thus such an analysis could contribute to the understanding, at the molecular level, of how these proteins perhaps participate in the regulation of the activity of the muscles in which they are present.

The present report is concerned with the results of a series of preliminary NMR experiments, obtained by proton spectroscopy at 100 and 270 MHz, on two parvalbumins (from hake and carp) and aiming at the visualization of conformational changes associated with the presence of a chemical denaturing agent, guanidinium chloride, with temperature variation, and with the removal of the firmly bound calcium ions. On the other hand, in connection with the determination of the primary structure of parvalbumins [9], the use of the NMR technique has been explored as a convenient probe for establishing the presence, directly in the proteins themselves, of an acetyl N-terminal blocking group.

(*) Contribution n° 99 from the Département de Biochimie Macromoléculaire and n° 1 from the E.R.140. This investigation was supported in part by the Délégation Générale à la Recherche Scientifique et Technique, by the Fondation pour la Recherche Médicale Française and by the Centre International des Etudiants et Stagiaires.

(**) Permanent address : Instituto de Biologia Fundamental, Universidad Autonoma de Barcelona, Barcelona, Spain.

(***) Permanent address : Laboratoire de Calcul et Traitement de l'Information, U.S.T.L., 34000 Montpellier, France.

MATERIALS AND METHODS.

The major parvalbumins (p*I* 4.36) from hake (*Merluccius merluccius*) and one of the major parvalbumins (p*I* 4.25) from carp (*Cyprinus carpio*) were prepared as described elsewhere [10].

Calcium was removed through Sephadex G-25 filtration (1 × 30 cm acrylic plastic column for 10-15 mg protein, collection in polypropylene tubes) in the cold room, using a 0.005 M sodium EGTA (ethylene glycol bis-(β-amino ethylether) N,N'-tetraacetic acid) solution, pH 7.5, as eluent. Lyophilization of the protein peak afforded samples containing about 0.4 mole Ca/mole protein, as estimated by atomic absorption spectrometry, which were redissolved in D_2O, yielding protein solutions of concentration *ca* 0.0015 M, buffered at pD 7.5 with 0.012 M EGTA.

NMR spectra were recorded around 30°C using 5 mm precision tubes, under continuous wave (CW) conditions (frequency sweep) on a Varian HA-100 spectrometer equipped with a C.A.T. Varian C-1024 device to improve the signal to noise ratio (Laboratoire de RMN, Université des Sciences et Techniques du Languedoc, Montpellier), and under rf pulse conditions on a Bruker HX-270 spectrometer equipped with the Fourier transform (FT) technique and using a Nicolet 32 K computer (Spectrospin AG, Fällenden, Switzerland). Variable temperature experiments were carried out on the Varian HA-100 spectrometer using the V-6040 temperature-control device. NMR spectra using the CW mode were obtained in two distinct regions, that at lower field from + 6.0 to + 11.0 ppm and that at higher field from — 1.5 to + 3.5 ppm ; in the FT mode the spectral width, 12 ppm, covered the whole spectrum.

A known quantity of parvalbumin was dissolved in 0.5 ml D_2O (99.9 p. cent deuterium, C.E.A., Saclay) being 0.045 M in potassium phosphate and of pD = 7.6 (20°), yielding protein solutions of concentration 0.001 - 0.004 M. Guanidinium chloride (GnCl), dissolved in the buffer above so that a 6 M solution was obtained (515 mg/0.5 ml), was used as a denaturing agent in order to get reference chemical shifts in the absence of any tertiary structure contribution. The solvent signal was used as an homonuclear proton internal lock signal (*H*DO) during the CW experiments and as an heteronuclear deuteron internal lock signal (H*D*O) during the FT experiments. Chemical shifts are quoted in ppm and positive numbers indicate shifts to low field from the reference resonance sodium 2,2-dimethyl - 2 - silapentane - 5 - sulfonate (DSS). The sodium salt was avoided from the protein solutions to prevent conformational disturbances and signal overlapping. The calibration of spectra was obtained using a solution of 0.01 M DSS in D_2O ($\Delta = \delta_{HDO} - \delta_{DSS} = 4.69 \pm 0.02$ ppm) and in 6 M guanidinium chloride-D_2O ($\Delta' = \delta_{HDO} - \delta_{DSS} = 5.00 \pm 0.02$ ppm) at *ca* 30°. Chemical shifts ($\delta_{obs,\ 30°}$) are defined at ± 0.03 ppm. It has been verified that the methyl signal position of DSS is not concentration dependent from 0.5×10^{-2} to 2.0×10^{-2} M DSS for D_2O as well as for 6 M GnCl-D_2O solutions.

All calculations were performed using an IBM 360-44 computer (Université des Sciences et Techniques du Languedoc, Montpellier).

RESULTS AND DISCUSSION.

NMR spectra of the chemically denatured proteins (Fig. 1 and 2).

The well resolved singlet signals at 2.10 and 2.06 ppm in the spectra of the denatured hake and carp components, respectively, are attributed to the N-terminal blocking acetyl group, which has now been identified by chemical means also [9, 4]. The signals at 1.98 ppm refer to contaminant acetate present in the preparations, as can be ascertained by its increase when the spectrum is reexamined after addition of a small amount of ammonium acetate (fig. 1 b and 2 b). The singlet at 2.04 ppm in the hake spectrum (fig. 1) can be ascribed to the S-CH_3 group of Met 105 ; sharp resonances of methionine methyl protons have been predicted to appear at 2.06 ppm [11] and have also been observed in that region for thermally denatured hen lysozyme [11]. This signal is absent in the spectrum of the carp component (fig. 2) which is devoid of Met and has a Leu at position 105 [4].

Enzymatic cleavage of the performic acid oxidized derivative of the hake parvalbumin affords the N-terminal nonadecapeptide T_1 [12] which includes the N-terminal acetyl group but not the S-CH_3 of methionine 105 ; the NMR spectrum (fig. 1d) of this peptide shows the presence of a singlet at + 2.11 ppm which can be ascribed to the N-acetyl group (*).

(*) The spectrum was determined on a sample in D_2O containing 25 p. cent deuterated pyridine necessary for keeping the peptide in solution. Apart from specific solvatation effects, the whole spectrum is shifted upfield (*ca.* 0.3 ppm) because of the aromatic character of pyridine [13]. To avoid corrections due to these solvent effects, the signal corresponding to contaminant ammonium acetate was taken as an internal reference at 1.98 ppm. The position of the N-terminal acetyl group of peptide T_1 at 2.11 ppm is in agreement with the results of Cozzone and Marchis - Mouren (*FEBS-Lett.* (1970) 9, 341-344.).

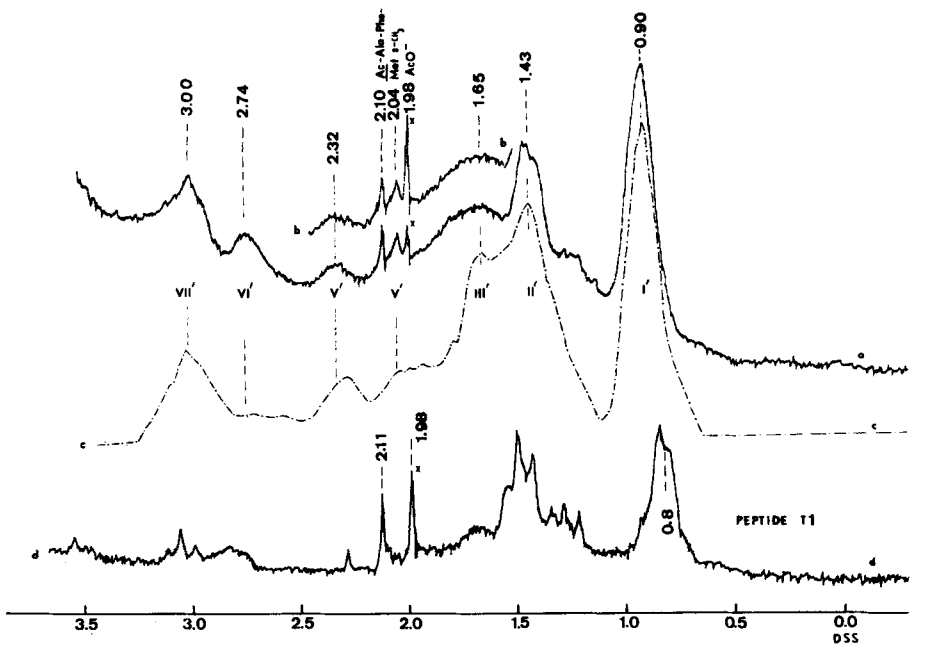

FIG. 1. — *100 MHz CW ^1H NMR spectrum (higher field region between 0.0 and + 3.5 ppm) at 27° C of the major hake parvalbumin in 6 M GnCl-D_2O ; protein : 8.35 × 10^{-4} M.*

a) 190 scans.

b) 177 scans ; 5 µl AcOH 0.1 M added : the acetate signal (1.98 ppm) is labelled x.

c) simulated spectrum according to the data or ref. 11 and to the amino acid composition (7) ; the N-terminal acetyl group is not included in the calculations.

d) 100 MHz spectrum of the N-terminal nonadecapeptide T_1 of the hake parvalbumin in C_5D_5N/D_2O (1/3, v/v).

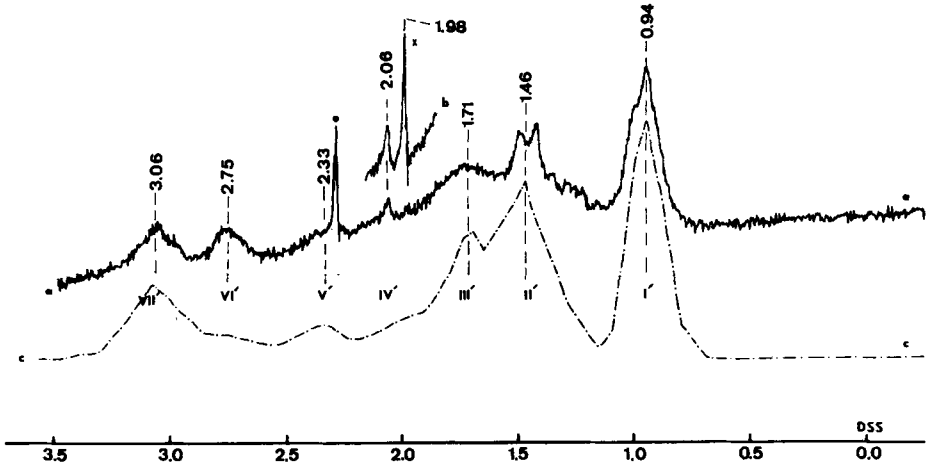

FIG. 2. — *100 MHz CW ^1H NMR spectrum at 27°C of the carp parvalbumin in 6 M GnCl-D_2O ; protein : 1 × 10^{-3} M.*

a) 85 scans ; o : impurity.

b) 219 scans ; 10 µl AcOH 0.1 M added : the acetate signal appears at 1.98 ppm and is labelled x.

c) simulated spectum according to the data of ref. 11 and to the amino acid composition (4) ; the N-terminal acetyl group is not included in the calculations.

As shown in figures 1 and 2, the denatured spectra have been predicted by simulation on the basis of composition only using the procedure of McDonald and Phillips [11]. In fact, the observed chemical shifts, $\delta_{obs,\ 30^\circ}$ (referred to 0.01 M DSS solutions), are larger than those reported in ref. 11 for proteins in random-coil configuration.

The chemical shifts observed ($\delta_{obs,\ 30^\circ}$) in the spectra of chemically denatured parvalbumins correspond to reference values in the absence of = 0.1 ppm) could be due essentially to the difference in temperature between the two cases, 30° (exp.) and 40° (calc.) (*). The actual chemical shifts reported in Figs 1 and 2 were adjusted using the relationship, $\delta_{calc} = \delta_{corr,\ 30^\circ} - 0.1$ ppm.

The present results, however, even established with the aid of only two low-molecular proteins, clearly demonstrate the essential validity of the procedure proposed by McDonald and Phillips [11]. Indeed, independently of the significant dif-

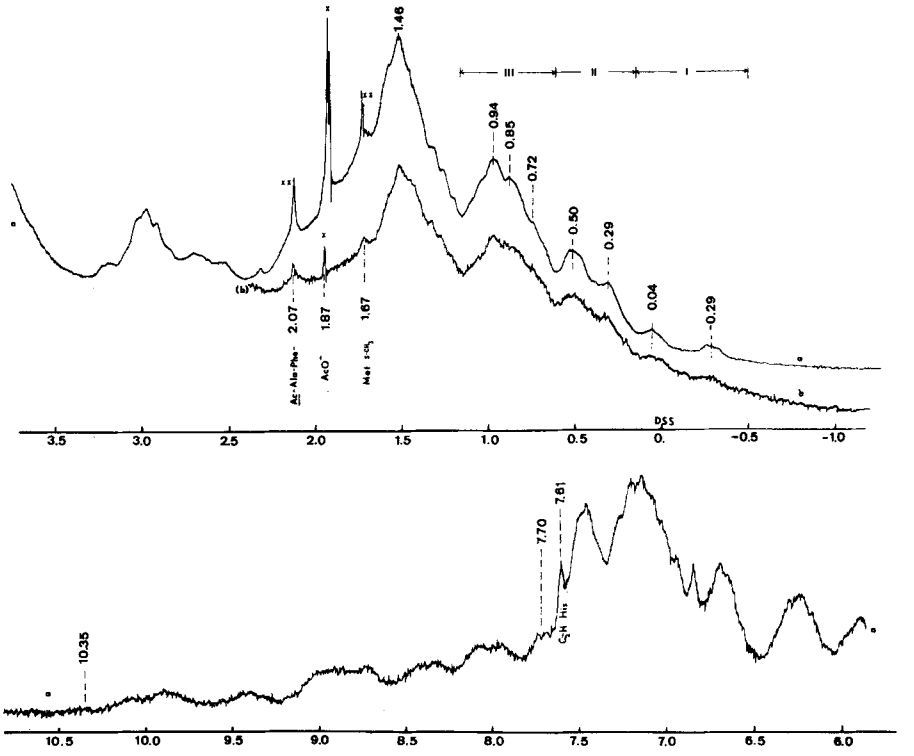

FIG. 3. — *100 MHz CW ^1H NMR spectrum at 27°C of the major hake parvalbumin in phosphate-D_2O buffer solution,* pD = 7.6 (a) protein : 4.3 × 10^{-3} M, (b) protein : 1.7 × 10^{-3} M after Sephadex G-25 gel filtration of sample a.

Upper part : region from — 1.0 to + 3.5 ppm. a) 82 scans (the strong signal x at 1.87 ppm corresponds to external acetate ion ; the two signals labelled xx are the corresponding spinning sidebands) ; b) 92 scans.

Lower part : a) region from + 5.8 to + 11.0 ppm ; 140 scans.

any tertiary structure contribution, provided they are corrected for the influence of GnCl, using the relationship, $\delta_{corr,\ 30^\circ} = \delta_{obs,\ 30^\circ} + a$ ($a = \Delta - \Delta' = -0.31$ ppm at 30°). For instance resonances corresponding to the methyl groups of Ileu, Leu and Val appear around + 1.30 ppm ($\delta_{obs,\ 30^\circ}$) ; the corrected value ($\delta_{corr,\ 30^\circ}$) is therefore about 0.99 ppm. Nevertheless, calculation of simulated spectra using the data of ref. 11 yields a value of + 0.89 ppm (δ_{calc}). The discrepancy between experimental and simulated spectra ($\delta_{corr,\ 30^\circ} - \delta_{calc}$ ferences in amino acid composition between the hake and the carp parvalbumins [7, 4], the simulated spectra in figures 1 and 2 both have a similar general shape and comprise clearly defined spectral regions I' (0.9 ppm), II' (1.4 ppm), III' (1.6 ppm), IV' (2.0 ppm), V' (2.3 ppm) VI' (2.7 ppm) and VII' (3.0 ppm) agreeing well with those of their experimental counterpart. Only for the reso-

(*) The observed difference appears in agreement with the chemical shift dependence of the water proton signal, which is about 0.01 ppm/°C.

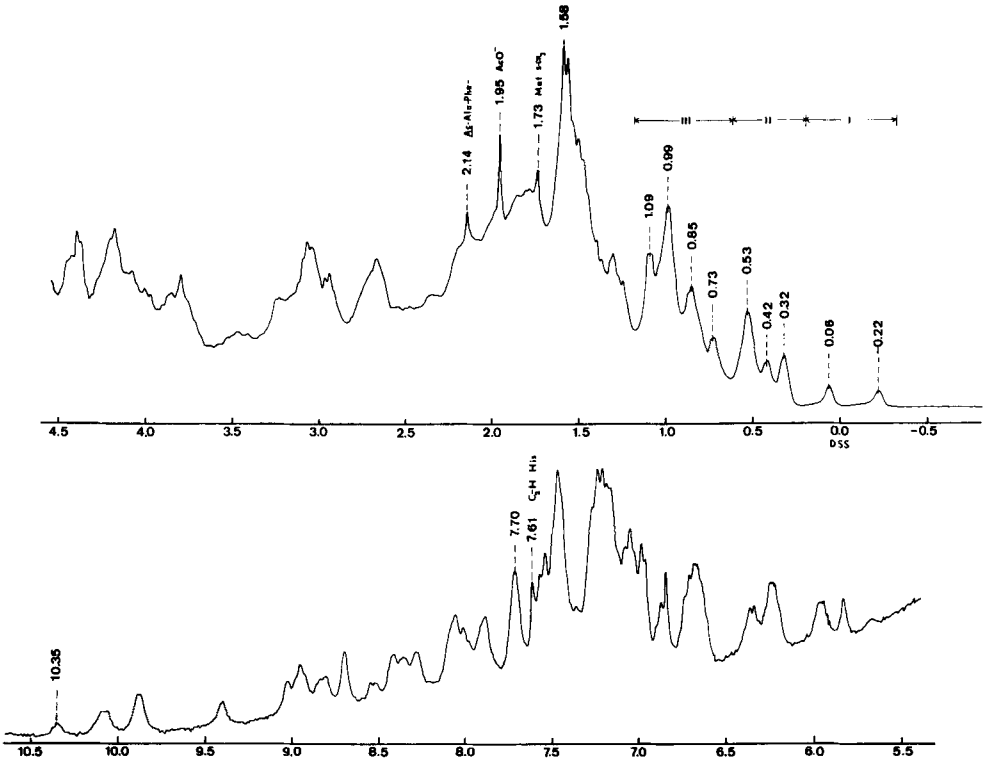

FIG. 4. — *270 MHz FT ^1H NMR spectrum at 31°C of the major hake parvalbumin in phosphate buffer-D_2O solution,* pD = 7.6 ; protein : 2.8 × 10^{-3} M ; pulse width 25 µsec, delay time 1.25 sec, 1800 scans.

Upper part : region from — 0.5 to + 4.5 ppm.
Lower part : region from + 5.5 to + 10.5 ppm.

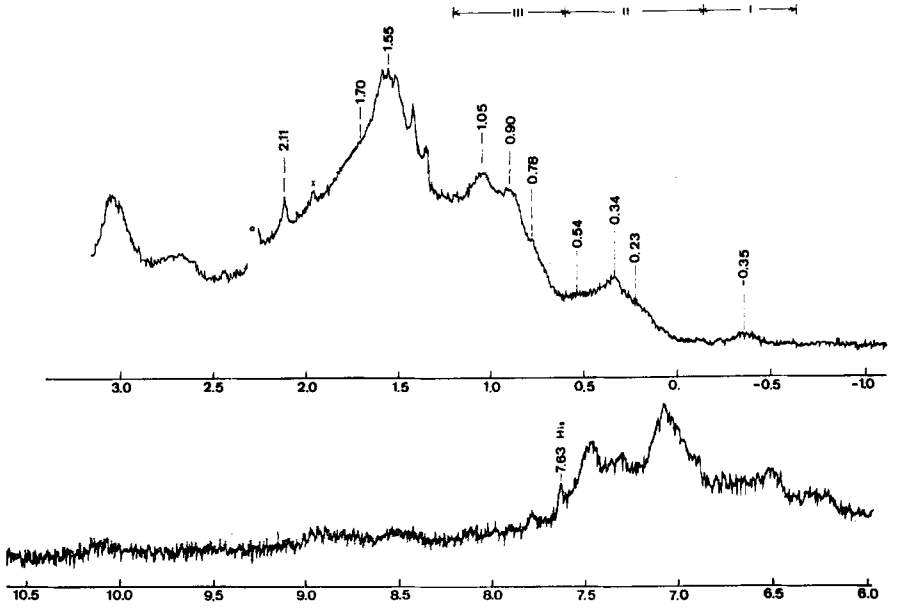

FIG. 5. — *100 MHz CW ^1H NMR spectrum at 27°C of the carp parvalbumin, in phosphate-D_2O buffer,* pD 7.6 ; x : external acetate ion ; o : impurity.

Upper part : region from — 1.0 to + 3.2 ppm ; 447 scans ; protein : 4.4 × 10^{-3} M.
Lower part : region from + 6.0 to + 10.5 ppm ; 437 scans ; protein : 1.95 × 10^{-3} M.

nance signals in zone VI', around 2.75 ppm, corresponding essentially to the Asp residues, is there a marked discrepancy in both cases between the two types of spectra. The observed differences in this region are certainly due to an overestimation of the signal half-width (55 Hz) of the Asp

the S-CH_3 group of the single methionine present in the hake component. Their positions are : N-acetyl : 2.14 ppm (hake, fig. 4) and 2.13 ppm (carp, fig. 6) ; histidine C_2-H : 7.61 ppm (hake, fig. 3 and 4) and 7.66 ppm (carp, fig. 6) ; methionine S-CH_3 : 1.73 ppm (hake, fig. 4).

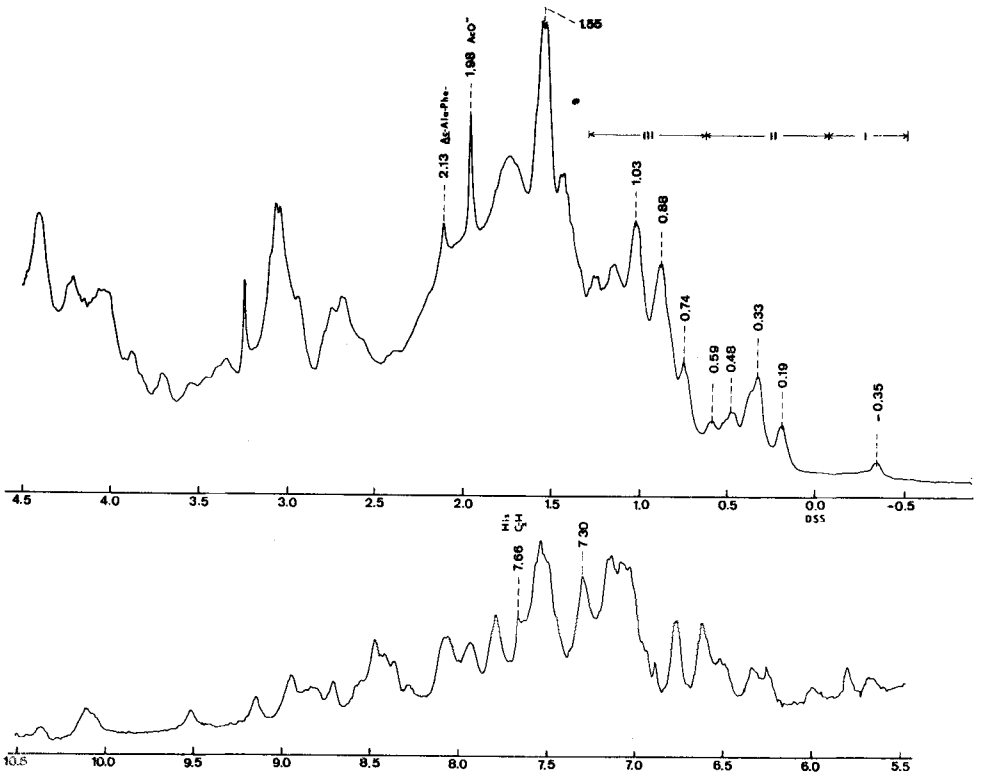

FIG. 6. — *270 MHz FT ^1H NMR spectrum at 31°C of the carp parvalbumin in phosphate buffer - D_2O solution*, pD = 7.6 ; protein : 2.76 × 10^{-3} M ; pulse width 25 μsec, delay time 1.25 sec, 1800 scans.

Upper part : region from — 0.5 to + 4.5 ppm.

Lower part : region from + 5.5 to + 10.5 ppm.

residue (β-CH_2 group) in the data of ref. 11. Parvalbumins, which have a high Asp content (11/108 residues and 14/108 residues for the hake and carp components examined, respectively [9, 4]), could actually be used for a good standardization of the Asp residue signal ; the NMR analysis of a large variety of parvalbumins will thus allow to obtain a precise value for its half-width.

NMR Spectra of the native proteins (fig. 3, 4, 5 and 6).

The signals referring to the N-terminal acetyl group still appear well resolved in the spectra of both the hake and the carp native proteins ; other signals also appear characteristically such as those of the C_2-H group of their single histidine and of

The general appearance of these spectra however, is quite different from those of the denatured proteins. A very large number of discrete signals is now observed (see table I) resulting from the effect of the various and specific molecular interactions in the native structures on the chemical shifts for each group of equivalent protons.

From the high Phe content (10/108 residues) for the hake and carp parvalbumins [7, 4] it could be predicted that hydrophobic interactions between the aromatic residues and the side chain of aliphatic residues, especially methyl groups, would be particularly strong and determinant in the cohesion of the whole molecule. As described by Nockolds *et al.* [4] this is indeed the case for the

carp parvalbumin where 18 non-polar aliphatic residues (7 Leu, 4 Ile, 3 Val and 3 Ala) are internal and lie in an aromatic environment of 8 internal Phe residues. An explanation for the shifts observed in the native proteins with respect to the denatured ones can therefore be attempted essentially on the basis of the magnetic perturbation due to the Phe residues, especially in the upfield part of the spectra. The large amplitude of the aromatic ring currents as compared to other diamagnetic anisotropy effects, makes it possible, in a first approximation, to take the influence only of the former into account when calculating the NMR spectrum of a protein of known tertiary structure.

The magnitude of the shielding due to a benzene ring has been predicted quantitatively by Johnson and Bovey [14] using the concept of ring current. The JB model has been satisfactorily applied to predict intramolecular shielding effects [15], as well as intermolecular effects, such as aromatic solvent effects [13].

TABLE I.

Upfield resonance in the NMR spectra (100 and 270 MHz) of native and carp parvalbumins. Chemical shifts are referred to internal DSS.

Region	Hake parvalbumin		Region	Carp parvalbumin	
	100 MHz	270 MHz		100 MHz	270 MHz
I	— 0.29 0.04	— 0.22 0.06	I	— 0.35	— 0.35
II	0.29 0.45 (S) 0.50	0.32 0.42 0.53	II	0.23 (S) 0.34 0.54	0.19 0.33 0.36 (S) 0.48 0.59
III	0.72 0.85 0.94 1.02	0.73 0.82 (S) 0.85 0.87 (S) 0.99 1.08 1.10	III	0.78 0.90 1.05	0.74 0.84 (S) 0.88 0.98 (S) 1.03 1.14 1.17 (S)
IV	1.17 (S) 1.23 (S) 1.29 (S) 1.40 (S) 1.46 1.52 (S)	1.12 (S) 1.24 1.30 1.32 (S) 1.37 1.39 1.45 (S) 1.47 1.50 1.53 (S) 1.56 1.58 1.61 (S)	IV	1.23 1.35 1.42 1.55	1.24 1.26 1.29 (S) 1.34 (S) 1.39 (S) 1.42 (S) 1.44 1.46 1.54 1.56
V	1.67 (a) 1.87 (b) 2.07 (c)	1.73 (a) 1.78 1.81 1.85 1.95 (b) 2.14 (c)	V	1.70 (S) 2.11 (c)	1.74 1.98 (b) 2.13 (c)

(a) -S-CH_3 signal of Met 105 of hake parvalbumin ; this signal is absent in the carp spectrum. (b) External acetate. (c) Acetyl N-terminal blocking group. (S) shoulder.

The JB model was applied to the carp parvalbumin using the known X-ray coordinates. The use of this model in the case of a protein containing only Phe as aromatic residues appears well justified on the basis of the electronic similarity between the aromatic ring of phenylalanine and that of benzene. A similar treatment was previously applied to lysozyme by Sternlicht and

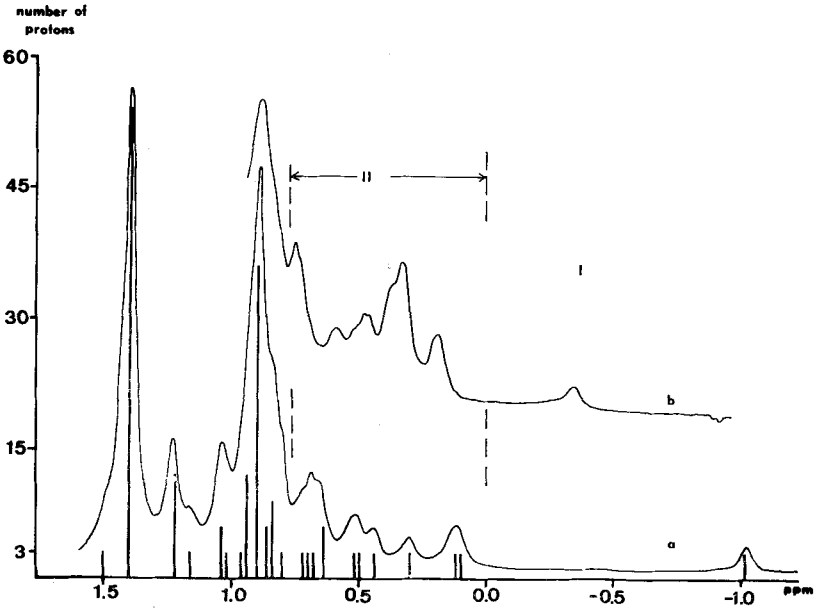

FIG. 7. — a) *Aliphatic simulated NMR spectra of the carp parvalbumin at 270 MHz including 63 methyl groups (from 5 Thr, 20 Ala, 5 Val, 9 Leu, 5Ile).* Vertical bars are proportional to the number of methyl groups with the same chemical shift ; a Lorentzian line shape was adopted with 15 Hz in half-width. b) *Relevant part of the experimental spectrum from figure 6.*

TABLE II.

Calculated increments for the methyl group major chemical shifts in the aliphatic residues of carp parvalbumin using the JB model.

Perturbing aromatic residues (Phe)	Perturbed aliphatic residue (a)		Calculated increments (in ppm) (b)	Chemical shifts in ppm (referred to DSS) (c)	
29	Ala	14 iH	— 0.77	+ 0.64	
29, 70 and 29, 30	Val	33 iH	— 0.80 and + 0.11	+ 0.13	+ 2.04
47, 102	Ile	58 i	— 0.39 and ± 0.00	+ 0.44	+ 0.83
66, 85 and 85	Leu	63 iH	— 0.79 and — 0.16	+ 0.10	+ 0.73
24, 29, 66, 70 and 24, 29	Leu	67 iH	— 0.24 and + 0.01	+ 0.65	+ 0.90
24 and 66	Leu	77 i	— 0.59 and + 0.01	+ 0.30	+ 0.90
85	Ile	97 i	— 0.31 and ± 0.00	+ 0.52	+ 0.83
47 and 57	Val	99 sH	— 0.25 and — 0.12	+ 0.68	+ 0.81
30, 102 and 30, 102	Val	106 iH	— 1.96 and — 0.43	— 1.03	+ 0.50

(a) Following ref. 4, i = internal residue, H = residue in α-helix region, s = residue at the surface of the protein.

(b) Calculated increments were obtained using a table of nuclear shielding values communicated to one of us by C. E. Johnson Jr. and F. A. Bovey in conjunction with ref. 14. The values of z and p (cylindrical coordinates : the z axis is normal to the plane of the aromatic ring at its center, the p axis lies in the plane of the ring) range from 0.00 to 4.00 (measured in ring radii 1.39 Å) in increments of 0.1. The proton positions of CH_2 and CH groups were determined by assuming tetrahedral bond angles, using X-ray coordinates of the carbon positions ; the methyl protons were assumed to be located at the center of the base of the cone formed by the freely rotating CH_3 group about the C-C bond, according to refs 15 and 16. The increments under 0.25 ppm have not been considered.

(c) The unperturbed chemical shifts correspond to those reported in ref. 11 (Ala : 1.41 ; Ile : 0.83 ; Leu : 0.89 ; Val : 0.93).

Wilson [16] although the aromatic perturbation included Trp and His residues (*).

It is seen from figure 7 that the calculations lead to the prediction of characteristic upfield shifts for the protons of a great number of the methyl groups of the carp parvalbumin. Twelve of these methyl signals should be shifted to a significant extent (more than 0.15 ppm, see table II) and they are expected to appear under regions I and II (as defined in table I) in the spec-

TABLE III.

Integration (number of protons) (a) of regions I and II in the spectra of the native carp and hake parvalbumins.

		I	II	N-acetyl
Carp	100 MHz (fig 5)	3.0	45-48	
	270 MHz (fig 6)	3.0	42	2.1 (b)
Hake	270 MHz (fig 4)	6.0 (c)	45	

(a) Integrations were obtained by planimetry.

(b) The exact surface of the N-acetyl singlet signal at 2.13 ppm is difficult to estimate accurately because of spectral overlapping with signals in the region V of the spectrum (fig. 6). Therefore the calculations were normalized to the surface of I which was chosen as corresponding to one methyl group.

(c) Region I in hake parvalbumin contains two methyl group signals (see table I).

trum of the native carp parvalbumin (fig. 6). The experimental spectrum (see figure 7, insert) actually agrees poorly with this prediction as far as resonance positions and total resonance shape are concerned. Nevertheless, the number of highly shifted signals is predicted correctly when the integration of regions I and II of the experimental spectrum is compared to that of the corresponding regions in the calculated spectrum (see table III).

The farthest experimental upfield signal (- 0.35 ppm, region I) can be ascribed to one of the methyl groups of Val 106, in spite of the fact that the calculated value is - 1.03 ppm. The absence of half-width change of this signal, when measured at either 100 or 270 MHz, ($\Delta\nu_{1/2}$ = 15 Hz in both cases), indeed points to it being a single methyl resonance. Region II, on the other hand, would include essentially 11 methyl resonances according to the calculations (Leu 63, Val 33, Leu 77, Ile 58, Val 106, Ile 97, Ala 14, Leu 67, Val 99, Ile 49 and Leu 63, in order of increasing chemical shift).

(*) The single His 26 of the carp parvalbumin has been excluded from the calculations ; this residue is not involved in interactions with aliphatic residues [4].

If the calculations are extended to methylene and methine groups, no significant upfield shifts result for these groups of protons. In the case of lysozyme [16], very upfield resonances due to aromatic interactions include methylene as well as methyl groups, a result which is thus markedly different from that obtained with the carp parvalbumin.

Although Nockolds *et al.* [4] reported no π-π stacking between the 8 internal Phe residues, their relative proximity suggests that there is a great magnetic interaction between them. The lower field spectrum of the carp parvalbumin (fig. 6) as well as of the hake parvalbumin (fig. 4) comprises intense signals between + 5.5 and + 6.5 ppm, a relatively upfield position for protons in proteins having no other aromatics than Phe (random coil chemical shift : + 7.26 ppm [11]) ; furthermore, there are no large downfield shifts above + 7.7 ppm (*). The observed upfield displacements thus certainly result from diamagnetic interactions between the Phe residues. In agreement with experiment, treatment of the 10 Phe residue system of the carp parvalbumin according to the JB model also affords very upfield shifts for the aromatic protons (between 5.0 and 6.5 ppm) and only small downfield shifts (between 7.5 and 7.7 ppm) (**). The region between 7.0 and 7.5 ppm, where the main protons are located in the experimental spectrum (fig. 6), is not well predicted, however. A strong line results at 7.26 ppm instead of the complex pattern obtained experimentally ; also, the intensity of this calculated line exceeds by far the single signal of moderate intensity which is observed at 7.30 ppm (fig. 6).

Several explanations for the observed quantitative discrepancies between experimental and calculated native spectra could be invoked :

1) The calculations in figure 7 only include the magnetic perturbation due to the Phe residues. Other contributions such as electric field and

(*) The spectra of the carp and hake parvalbumins dissolved in D_2O actually include resonances up to + 10.5 ppm ; however these resonances belong to NH-amide protons involved in hydrogen bonding and inaccessible to solvent (see below). When total exchange with deuterium has occurred no aromatic resonance appears at lower field than 7.7 ppm.

(**) This results from the calculations for the aromatic counter part according to the JB model and the X-ray coordinates, which allow to predict the chemical shifts for the 50 aromatic protons of the carp parvalbumin ; a simulated spectrum at 270 MHz was obtained with the aid of a LAOCN 3 program adapted to the case of the aromatic five spin system of the Phe residue and using standard spin-spin coupling constants for an alkyl monosubstituted benzene (J_{ortho} = 8.0 Hz ; J_{meta} = 2.0 Hz ; J_{para} = 0.5 Hz) as well as Lorentzian line shape with 15 Hz in half-width.

α-helix structure effects have not been considered in this first approach. Actually, the Sternlicht and Wilson's discussion on lysozyme leads to the conclusion that only small chemical shift changes would occur upon folding into regions of right-handed helices [16].

2) The structure in solution differs slightly from that in the crystal.

3) In the dissolved state, the equilibrium between rotamers of the side chains can be altered, as well as the kinetics of their interconversion. In this view, the two methyl groups of Val 106, for instance, would be located at two mean positions between the extreme calculated ones, yielding a better agreement with experiment (*). The existence of different rotamers was already inferred for the Phe residues of parvalbumins on the basis of optical methods [1], and seems also to be supported for Val residue 106 from the NMR spectral behaviour of the carp parvalbumin when temperature is allowed to vary (see below).

The observation of characteristic upfield displacements for a set of methyl, and aromatic (Phe) protons in the NMR spectrum of the native carp parvalbumin (Fig. 5 and 6), is essentially in agreement with the molecular configuration established by X-ray crystallography [4]. At the present level of accuracy in the NMR analysis, it is not possible to say if the structure in solution is really identical to that in the crystal. As far as the internal hydrophobic core (see ref. 4 and table II) of the protein is concerned, this seems to be the case. In the same order of ideas, it has been established on quite different experimental evidence that the structure of a globular protein, such as an enzyme, appears to be the « same » in aqueous solution and in the wet crystalline state [17]. Even in this case, the criterion of identity of the molecule in both states can only be specified to the resolution of small fractions of a chemical bond length [17]. It must be emphasized that slight changes of coordinates (± 0.2 Å) between the crystalline (X-ray) and the dissolved (NMR) states would afford noticeable chemical shift changes up to 0.2 - 0.3 ppm, so that, in the present situation, only a qualitative or at least semi-quantitative agreement could be expected when comparing the results of NMR and X-ray crystallography.

Nevertheless, characteristic tertiary structure features of the carp parvalbumin, involving aliphatic-aromatic interactions are well reflected at the magnetic level on the NMR spectra : 1) interactions between near residues. In particular, interactions between an aromatic residue located at position n in an α-helix, and an aliphatic residue at position n ± 4 (or 5) of the same helix, such as between Phe 70 and Leu 67 and between Phe 102 and Val 106, are clearly visible. Such a regular alternance of non-polar residues appears as a characteristic of α-helical segments included in globular proteins, as shown by the results of Perutz *et al.* for hemoglobin [18]. 2) interactions between distant residues. Among these interactions, those involving elements of separate helical segments are of particular interest, as they could play a predominant role in the maintenance of the tertiary structure. The interactions Leu 67-Phe 29, Val 33-Phe 70 and Val 106-Phe 30 are of this type. The last two are probably quite strong as shown by the corresponding upfield increments (see table II). The Val 106 - Phe 30 interaction, which involves the two helical segments B and F (*), could play an important role in the function of the molecule, as the F helix includes the carboxyl terminal part of the chain and is adjacent to the EF (*) region (residues 90 to 101) which is one of the two calcium binding sites [4].

The similarity of the methyl high field regions in the NMR spectra of the hake and carp parvalbumins (Fig. 3-4, and Fig. 5-6, respectively), added to the fact that almost (**) all the aliphatic and aromatic residues involving an important magnetic interaction (see table II) are invariant in the primary structure of the two proteins suggests that some of the resonance signals of the hake parvalbumin can be identified in spite of the fact that no atomic coordinates are available for this protein. The signal at — 0.22 ppm certainly represents one of the two methyls of hake's Val 106 which would lie in a chemical environment very similar to that observed in the carp parvalbumin (presence of the B and F helical segments). The other upfield resonance in region I, at + 0.06 ppm (3 protons), is tentatively attributed to one methyl of Leu 63. The observation of two upfield resolved

(*) These appear to be -1.03 and + 0.70 ppm on the basis of calculations including three rotamers of Val 106 through rotation around the Cα —Cβ bond ; if the internal energy of these rotamers differs markedly, the two methyl groups of Val 106 would appear as two distinct signals even in the case of a rapid interconversion.

(*) The X-ray crystallographic results [4] established the presence in parvalbumins, of α-helical regions named A, B, C, D, E and F ; the non helical peptide regions between two helical regions are named by two letters (EF, e.g., corresponds to the peptide region between helices E and F).

(**) In hake [9] Val 33 of carp is replaced by Ile, and Ile 58 by Val. All the other elements of Table II remain unchanged.

signals in the hake spectrum, instead of one in the carp spectrum, is perhaps to be related to a local difference in the two structures, in particular regarding the spatial relationship between Leu 63, Phe 66 and Phe 85. However, the two proteins which differ by 27 amino acid substitutions, have in all other respects very similar NMR spectra, which point to their having also a similar tertiary structure. Integration of the upfield region of the 270 MHz spectrum of the hake parvalbumin is in agreement with this assumption (see table III).

It is interesting to notice that all the residues which give rise to the upfield regions I and II in the spectrum of the carp parvalbumin (perturbed aliphatic and perturbing aromatic residues of table II) are not only unchanged in the hake's primary structure but also in that of four other parvalbumins whose sequence is partly known (J. F. Pechère, J. P. Capony and J. Demaille, in preparation). This invariance is quite remarkable and suggests that the aliphatic-aromatic interactions are essential in the cohesion of the tertiary structure of parvalbumins.

The observed upfield shifts in the NMR spectra are actually in agreement with a contact where the methyl group of the aliphatic residue lies above the plane of the aromatic ring, in the z direction (see legend of table II). According to the thermodynamic treatment of Nemethy and Scheraga [19], this configuration leads to a stabilization of the interacting system. It is also very remarkable to observe that although the Phe content of parvalbumins is a relatively high one, the Phe residues occupy very separate positions in the sequences ; only one cluster is observed (doublet in positions 29-30). Such a configuration appears more suitable for an increased number of aliphatic-aromatic interactions to take place over the entire chain length than a more clustered one.

Although the hydrophobic interactions are certainly important in the cohesion of the parvalbumin structures, it must be remembered that these proteins belong to the class of globular proteins, where the stability results from a balance between many interactions (dipolar interactions, hydrogen bonding, van der Waals forces). The role of hydrogen bonding in the tertiary structure has been discussed by Kretsinger *et al.* [20] in their X-ray study of the carp parvalbumin.

The carp protein contains 178 exchangeable hydrogens, 108 peptide-NH hydrogens and 70 NH and OH hydrogens from Asp, Asn, Glu, Gln, Lys, Arg, Thr and Ser side chains. The last category corresponds to rapid solvent exchangeable hydrogens, particularly in the case of the carp parvalbumin where almost all polar side chain hydrogens (65 out of 70) are situated at the surface of the molecule [20]. Of the 108 peptide-amide hydrogens, on the other hand, 29 are exposed to solvent, 10 are bound to side chain oxygens [20] and these would therefore also exchange very rapidly. The above considerations thus yield a number of 104 protons (65 + 29 + 10) which are exchanged against deuterons when dissolving the protein into D_2O. In contrast, the remaining 74 protons, inaccessible to solvent and involved in the helical structures and internal hydrogen bonding, could have very low exchange rates against D_2O, as it has been noticed in the native conformation of other proteins [21, 22, 23]. The observed spectrum of the carp parvalbumin (fig. 6) in D_2O indeed seems to reflect the existence of differential exchange rates of the mobile protons of the molecule, a situation which is clearly apparent in the hake spectrum (fig. 3 and 4) from the behaviour of the signals at 7.70 and 10.35 ppm. The integration of the observed signals also suggests the presence of protons partially exchanged with deuterium.

Thermal denaturation of the carp parvalbumin.

The spectra reported in figure 8 show definite alterations of the tertiary structure of the carp parvalbumin when varying the temperature between 27° and 73°. At the highest temperature, the NMR spectrum is very characteristic of a denatured state, and appears very similar to that of the chemically denatured state of figure 2. However, precise differences appear which could be related, among others, to effects due to the difference in temperature between both spectra. Region I' in figure 8 (73°) is composed of two signals (0.95 and 1.02 ppm) ; from the theoretical predictions [11] based on the amino acid composition, the latter certainly corresponds to the Val residues. In the chemically denatured spectrum (fig. 2) only a shoulder is observed at this position resulting from a poorer resolution (chemical shifts and half-widths of signals) between the Val- and Leu-Ile-methyl signals. Another characteristic difference between the two kinds of spectra is the appearance of resolved spin-spin coupling multiplets at high temperature because of half-width changes ; this occurs in regions II' (intense doublet, centered at 1.48 ppm, J = 7 Hz, methyl groups of Ala residues), and in region VII' (triplet centered at 3.08 ppm, J $\simeq$ 7 Hz of the ε-CH_2 groups of Lys residues).

The shape of each spectrum in figure 8 can be readily understood as the sum of two spectra, one corresponding to the native state, the other to a

denatured state defined by what is observed at 73°. This is so because the interconversion of both species is a slow process on the NMR time scale

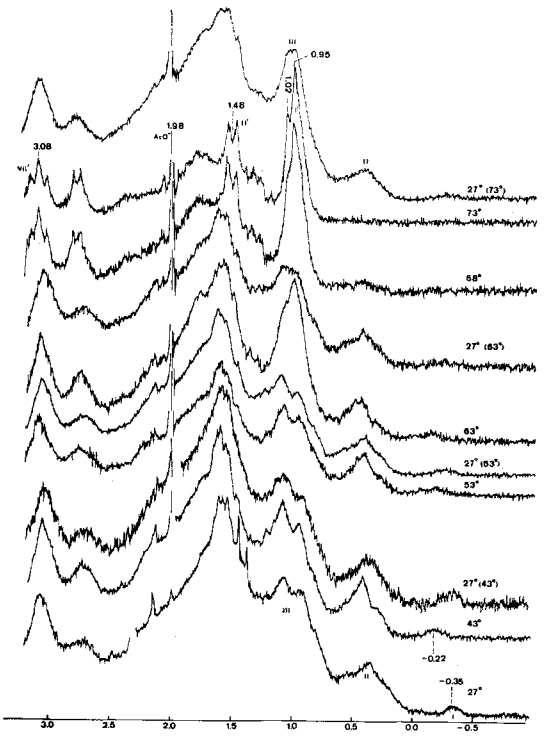

FIG. 8. — *100 MHz CW ^1H NMR spectra (high-field part) of the carp parvalbumin (protein : 2.9 × 10^{-3} M) in phosphate - D_2O buffer*, pD = 7.5 (*ca* 20°) at different temperatures.

The reference spectrum 27° corresponds to the sample of Fig. 5 at a lower signal/noise ratio (214 scans). The symbol 27° (T), T = 43, 53 and 63, means that the spectrum has been recorded at 27° after heating the sample at the temperature value reported between brackets ; the cooling period always reached several hours.

Chemical shifts are quoted in ppm/DSS but are referred to the methyl resonance of ammonium aceate which was chosen invariant at 1.98 ppm/DSS all through the temperature variation ; no temperature corrections have been applied for this reference system.

a melting temperature (temperature at half denaturation) of about 65°. It must be emphasized that the observed transition involves essentially hydrophobic residues and therefore could be representative of a melting process associated with the internal elements of the protein.

TABLE IV.

Thermal denaturation of carp parvalbumin under conditions of figure 8.

Temp. °C	P. cent denatured
27	0
43	0
53	4
63	38
68	77
73	95–100

The experiments involving a cycle of heating and cooling reported in fig. 8 also clearly establish the existence of a reversibility between the denatured state and the native state, all through the thermal transition. The reverted spectra,

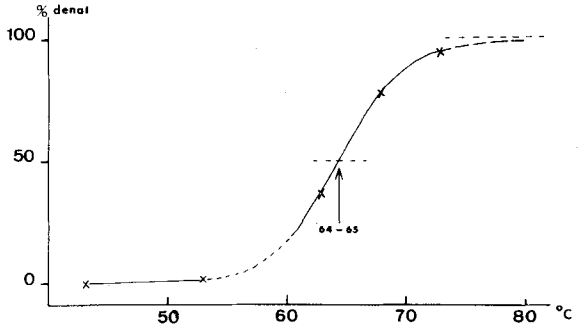

FIG. 9. — *Thermal denaturation curve of the carp parvalbumin under the conditions described in fig. 8.* The estimation of p. cent denat. is documented in the Appendix and in table IV.

[22]. However, because of the limitations of the NMR method itself, there is no definite proof that other intermediate conformations could not exist.

The results of calculations based on the simple two-state hypothesis and using the relationship,

$$\text{p. cent denat.} = \frac{S - 1.32 \times b}{S - 0.25 \times b} \times 100$$

given in the Appendix, are summarized at table IV, and in figure 9.

The thermal denaturation curve (fig. 9) is characteristic of a cooperative melting process, with however show some differences with their direct counterpart (27°) which could imply incomplete refolding ; the difference is particularly noticeable when comparing regions III (+ 0.6 at + 1.2 ppm) of the two native states, 27° and 27° (73°) in figure 8.

A very interesting feature in the NMR spectra of carp parvalbumin examined under varying temperature is the shift of the upfield methyl signal of Val 106 (27°, — 0.35 ppm ; 43°, — 0.22 ppm ; 53°, — 0.24 ppm ; 63°, — 0.20 ppm).

The main shift occurs between 27° and 43° when denaturation has not yet occurred ; chemical shifts as well as half-width changes are observed in that range of temperature. These shifts might be related to the existence of different rotamers for residue side chains as has already been discussed to that of the native protein (Fig. 5) and a comparison with that of the chemically denatured protein (Fig. 2) suggests that, concomittantly to the removal of the calcium ions, a conformation has been reached which is very similar to that of the denatured state.

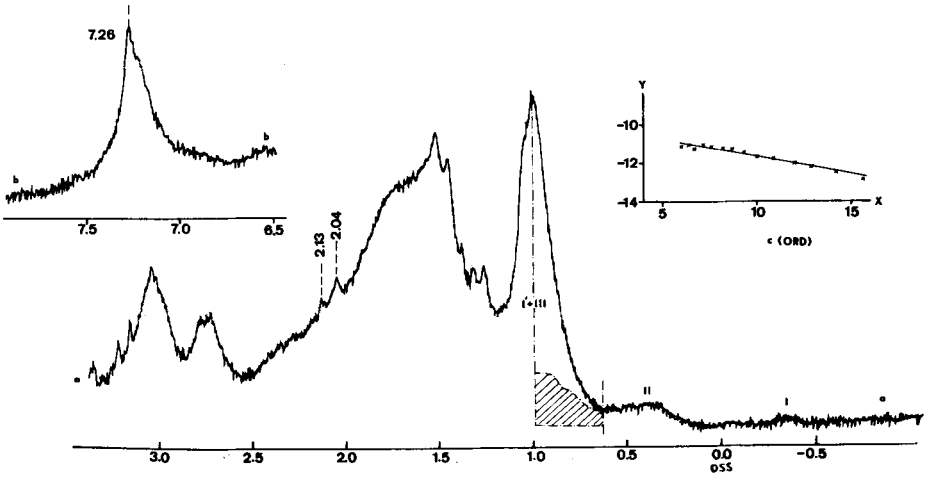

FIG. 10. — *100 MHz CW ^1H NMR spectrum at 27°C of the carp parvalbumin* (protein : 1.57×10^{-3} M) at low calcium ion content (*ca* 0.4 Ca^{++}/mole protein) in the presence of Na_2-EGTA 3.66×10^{-2} M. a) region from — 1.0 to + 3.5 ppm, 131 scans ; b) region from + 6.5 to + 8.0 ppm, 147 scans ; c) Moffitt-plot of the ORD measurements obtained with the NMR sample using a 0.1 cm optical path cell, at *ca* 21°C (the coordinates X and Y are defined in the same way as in fig. 2 of ref. 1).

above in relation to Val 106, and whose relative populations would change with varying temperature. The downfield shift of the methyl signal of Val 106 when raising the temperature is in agreement with such an interpretation. However, less localized conformational changes could happen, as the temperature is raised, which could affect to some extent the resonance positions of the Val 106 methyl groups ; if the two α-helix segments B and F were subjected even to a small relative displacement, the magnetic perturbation between Phe 30 and Val 106 (see table II) would be altered, leading to a chemical shift change for the methyl groups of the latter residue.

Ca-dependence of the spectra.

Of particular interest, now, is the analysis of the NMR spectra of the EGTA-treated carp parvalbumin, which establishes that important conformational changes do occur when the Ca^{++} ions are removed from the protein.

Fig. 10 depicts the NMR spectrum of a sample of the carp parvalbumin whose calcium content had been lowered to about 0.4 mole/mole protein. This spectrum is very much altered with respect

However, in the sample examined, resonances at higher field in the I and II regions are still present. This probably indicates that the present spectrum actually corresponds to a mixture of the native and of the denatured state. The presence of two N-acetyl singlets at 2.04 ppm (denatured state, to be compared with 2.06 ppm in Fig. 2) and 2.13 ppm (native state to be compared with 2.13 ppm in Table I), is consistent with this interpretation, their discreteness being indicative that the two species are not interconverting (or at least not at an appreciable rate), and are thus present in a definite proportion. Thanks to the fact that there is no spectral overlapping of the two states in the I and II regions, it is actually possible to perform an analysis of the spectrum reported in fig. 10 into its two components. Figure 11 depicts the NMR spectrum thus inferred to correspond to the calcium-free protein. This simulated spectrum is very similar to that which was obtained after chemical denaturation (fig. 2). Yet some quantitative differences are clearly apparent ; in particular zones II' and III' are more intense in the simulated calcium-free spectrum. This could be due either to some inaccuracy in the numerical

analysis, or to molecular properties inherent to the calcium-free state (local conformational differences, intermolecular effects such as aggregation (*)).

According to these results, complete removal of the calcium ions from the carp parvalbumin totally converts the native protein into a denatured one which is thus practically identical to that

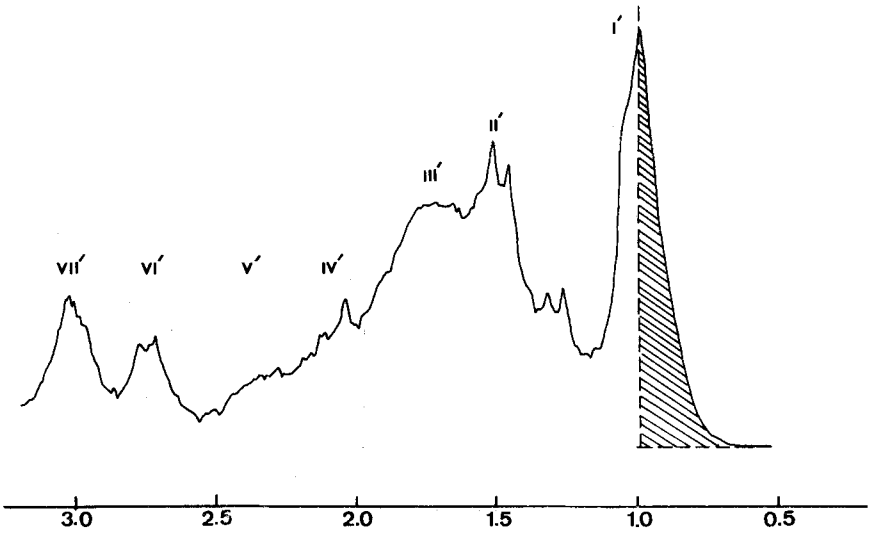

FIG. 11. — *Simulated NMR spectrum at 100 MHz of the fully Ca^{++}-freed carp parvalbumin.* This spectrum (SS) has been obtained using the data of fig. 9 (partially denatured spectrum, PDS) and fig. 4 (native spectrum, NS) : (SS) = (PDS) — P × (NS). P corresponds to the native state percentage (see text).

An estimation of the relative contribution of the native and calcium-free species to the spectrum of figure 10 can be obtained from a comparative integration of regions I' and II as described in the Appendix. It is then found that the contribution of both species lies around 20 p. cent native and 80 p. cent denatured. An independent check on this estimation is provided by ORD-measurements carried out as previously described [1] on the sample which gave the NMR spectrum of figure 10. These yield a value of 10 p. cent α-helix, instead of *ca* 50 p. cent for the fully native state [2, 4], from which the respective contribution of the native and denatured states can also be estimated to lie around 20 p. cent and 80 p. cent, in good agreement with what is found from the NMR integrals. Finally, the spectrum of figure 10 comprises an aromatic region with a single signal centered at 7.26 ppm in conformity with what is usually found with denatured proteins and with the theoretical predictions that can be made, on the above basis, by using the procedure of ref. 11.

(*) Calcium-freed parvalbumins have a strong tendency to aggregate, a situation which e.g. manifests itself by an important contribution of scattering to the absorption of light.

obtained by chemical denaturation (GnCl) or thermal denaturation. The finding of the 20 p. cent native form in the instance above would then merely result from the presence of about 0.4 moles Ca/mole protein in the sample.

From these experiments, it appears that the strongly bound calcium ions in parvalbumins play a determinant role in the maintenance of the native tertiary structure of these proteins. A confirmation of this conclusion is to be found in the observation that if the GnCl-denatured carp parvalbumin is filtered through Sephadex G-25 to remove the denaturing agent, the spectrum of the sample obtained is generally best interpreted, again, as resulting from a mixture of denatured and native molecules. Only if such a sample is dialyzed for some time *vs* diluted buffer without particular precautions, is the native spectrum unmistakebly reestablished, probably as a result of the progressive complete reloading of the sample with calcium picked up from the tubing and the glassware used during dialysis. Such a process is actually strongly reminiscent of the slow renaturation experiments which have been conducted successfully with many enzymes, such as ribonuclease or amylase [24, 25].

If the strongly bound calcium ions of parvalbumins are structurally required to preserve the integrity of their native structure, however, it would imply that their possible physiological function cannot be associated with a complete exchange of the metal ions. It is conceivable that, *in vivo*, because of association with another molecule which could also act as an effector, the denaturation concomittant to complete calcium removal is prevented. The possibility remains, nevertheless, that, in the cell, parvalbumins either release only part of their firmly bound calcium or that their activity is associated with the sites of lower affinity for the ion [8]. In this respect, it is interesting that preliminary experiments seem to indicate that the calcium content of parvalbumins can be lowered appreciably without alteration of the major characteristics of the conformation of the fully loaded state, and that, on the other hand, the chemical shifts of some signals (C_2-H of His, S-CH_3 of Met in the hake parvalbumin) are sensitive to calcium in excess of 2 moles/mole protein.

CONCLUSIONS.

The important chemical shift changes observed in the NMR spectra of parvalbumins in function of their conformational state suggest that this technique is a very valuable one for detecting and identifying such changes under varied conditions. Actually, the great solubility and the small size, as well as dominant role of the simple hydrophobic interactions which form the basis of the internal cohesion of these proteins and which can be well interpreted theoretically, all contribute to make parvalbumins a material of choice for NMR studies, especially as so much detailed information is available on their primary and on their tertiary structure.

The conformational changes associated with the specific Ca-binding properties of parvalbumins undoubtedly are among the most interesting aspects of such studies. The denaturation observed here concomittantly to complete calcium removal should be considered only as a first result to be supplemented by many future investigations. However, an appreciation of the technical conditions under which a more precise and quantitative analysis of the NMR spectra of parvalbumins can be conducted has thus been gained.

It is therefore reasonable to hope that a detailed investigation of the mechanism of calcium release and capture by parvalbumins can be successfully conducted by NMR spectroscopy. An extension and more gradual use of the techniques employed in the present work, coupled with CD experiments [1] should provide details about the changes in the general architecture of the molecule concomittant to such a process. On other hand, the use of paramagnetic shifts reagents, such as lanthanides, would be helpful in detecting conformational changes around the Ca^{2+} binding sites. Work is now in progress along these different lines.

Acknowledgments.

The kind communication of atomic coordinates of the carp parvalbumin by Dr. R. H. Kretsinger, Dept. of Biology, University of Virginia, is gratefully acknowledged. Drs. H. P. Kellerhals and W. Schittenhelm helped greatly by permitting the use of their supraconducting spectrometer Bruker HX-270. Mr. Ch. Gauffier, Laboratoire de RMN, USTL, Montpellier, is to be thanked for its efficient cooperation in the recording of the spectra. Dr. A. Fruchier, USTL, Montpellier, kindly provived a version of the LAOCN 3 NMR program.

Appendix. NMR integrations.

The spectra reported in figure 8 and figure 10 allow an estimation of the percentage of denaturation (p. cent denat.) of the carp parvalbumin under condition of heating or low calcium content.

The 100 MHz spectrum of figure 10 will be chosen as an example of quantitative analysis. Region I (surface a) and region II (surface b) belong exclusively to the native state ; if we define by c the surface under region I' (between 0.64 and 1.00 ppm ; dashed surface in figure 10) also belonging to the native state, it is known that c/b = 1.47 according to the data of Fig. 5 (upper part) ; furthermore, b represents *ca* 48 protons (b/a = 16, according to table III, carp, 100 MHz), so that c represents 48 × 1.47 = 70 protons.

In the completely denatured state (see figure 11 as well as figure 2 and 8) region I' essentially includes all the methyl groups of Leu (9), Ile (5) and Val (5) of the carp parvalbumin, i.e. 38 methyl groups ; if we define by d the high-field half surface of the corresponding signal of the spectrum (dashed surface in figure 11), this surface represents 57 protons with a good precision. Indeed the simulation according to ref. 11 (see figure 2c) indicates that in the high-field region of the spectrum there is no contribution of other protons than those of the methyl groups of Leu, Ile and Val.

In the partially denatured spectrum of Fig. 10 the surface d can be obtained by measuring the

whole surface S between 0.64 and 1.00 ppm, $d = S - c = S - b \times 1.47$.

Under these conditions, p. cent denat. $= \frac{S - 1.47 \times b}{S - 0.28 \times b} \times 100$, for the experiments dealing with calcium removal ; e.g. p. cent denat. = 79 for the sample of figure 10.

In the case of the thermal denaturation (fig. 8), p. cent denat. $= \frac{S - 1.32 \times b}{S - 0.25\ b} \times 100$.

RÉSUMÉ.

La spectroscopie RMN (résonance protonique à 100 et 270 MHz) a été utilisée pour observer des changements conformationnels dans deux parvalbumines musculaires en provenance du merlus (*Merluccius merluccius*) et de la carpe (*Cyprinus carpio*) lors du passage de l'état natif à l'état dénaturé (chlorure de guanidinium 6M ou chauffage) ou à celui obtenu après enlèvement presque complet des ions calcium. De ces observations il résulte que cette dernière opération conduit à une conformation très semblable à celle qui est obtenue après dénaturation chimique ou thermique. D'autre part, le spectre RMN de la parvalbumine de carpe native peut être interprété sur la base des données obtenues ailleurs lors d'une étude aux rayons X des cristaux de cette protéine. Certains aspects de la structure primaire de ces parvalbumines, en particulier la présence d'un résidu acétylé N-terminal, ont également été examinés à l'aide de la technique de RMN.

REFERENCES.

1. Parello, J. & Pechère, J.-F. (1971) *Biochim.*, 53, 1079-1083.
2. Rao, K. S. P. B. & Gerday, C. (1973) *Comp. Biochem. Physiol.*, **44 B**, 1113-1125.
3. Kretsinger, R. H., Nockolds, C. E., Coffee, C. J. & Bradshaw, R. A. (1971) *Cold Spring Harbor Symposium*, **36**, 217-220.
4. Nockolds, C. E., Kretsinger, R. H., Coffee, C. J. & Bradshaw, R. A. (1972) *Proc. Natl. Acad. Sci.*, **69**, 581-584.
5. Konosu, S., Hamoir, G. & Pechère, J.-F. (1965) *Biochem. J.*, **96**, 98-112.
6. Pechère, J.-F. (1968) *Comp. Biochem. Physiol.*, **24**, 289-295.
7. Pechère, J.-F., Capony, J.-P. & Rydèn, L. (1971) *Eur. J. Biochem.*, **23**, 421-428.
8. Benzonana, G., Capony, J.-P. & Pechère, J.-F. (1972) *Biochim. Biophys. Acta*, **278**, 110-116.
9. Capony, J.-P., Rydèn, L., Demaille, J. & Pechère, J.-F. (1973) *Europ. J. Biochem.*, **32**, 97-108.
10. Pechère, J.-F., Demaille, J. & Capony, J.-P. (1971) *Biochim. Biophys. Acta*, **236**, 391-408.
11. McDonald, C. C. & Phillips, W. D. (1969) *J. Am. Chem. Soc.*, **91**, 1513-1521.
12. Capony, J.-P. & Pechère, J.-F. (1973) *Europ. J. Biochem.*, **32**, 88-96.
13. Lazlo, P. (1967) *Progr. NMR Spectroscopy*, **3**, 231-402.
14. Johnson, C. E. Jr. & Bovey, F. A. (1958) *J. Chem. Phys.*, **29**, 1012-1014.
15. Durand, P., Parello, J., Buu-Hoi, N. P. & Alais, L. (1963) *Bull. Soc. Chim.*, 2438-2441.
16. Sternlicht, H. & Wilson, D. (1967) *Biochem.*, **6**, 2881-2892.
17. Rossi, G. L. & Bernhard, S. A. (1970) *J. Mol. Biol.*, **49**, 85-91.
18. Perutz, M. F., Kendrew, J. C. & Watson, H. C. (1965) *J. Mol. Biol.*, **13**, 669-678.
19. Nemethy, G. & Scheraga, H. A. (1962) *J. Phys. Chem.*, **66**, 1773-1789.
20. Kretsinger, R. H. & Nockolds, C. E. (1973) *J. Biol. Chem.*, **248**, 3313-3326.
21. Hvidt, A. & Nielsen, S. O. (1966) *Adv. Protein Chem.*, **31**, 287-386.
22. McDonald, C. C. & Phillips, W. D. (1970) Biological Macromolecules, Marcel Dekker, Inc., New York, **4**, 1-48.
23. Karplus, S., Snyder, G. H. & Sykes, B. D. (1973) *Biochem.*, **12**, 1323-1329.
24. Anfinsen, C. B. (1967) *Harvey Lect.*, **61**, 95-116.
25. Fukushi, T. & Isemura, T. (1968) *J. Biochem. (Tokyo)*, **64**, 283-292.