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Parvalbumins from the lungfish (*Protopterus dolloi*).

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Résumé.

Cinq parvalbumines ont été purifiées à partir des muscles blancs du *Protopterus dolloi* : les propriétés physico-chimiques de ces composants sont analogues à celles de cette famille de protéine. Elles se divisent en deux groupes distincts caractérisés par leur composition en acides aminés, leur résidu C-terminal, leur carte peptidique et leur réactivité immunochimique.

Les muscles rouges de ce poisson y compris le muscle cardiaque contiennent des parvalbumines en quantité plus faible de même que le cerveau et les reins.

Summary.

Five parvalbumins have been isolated from the white muscles of the lungfish. They can be divided into two sub families showing typical amino acid compositions, C-terminal amino acid residues, peptide maps and immuno-reactivity. The red muscles including the cardiac muscle also contain parvalbumins in amounts roughly inversely related to the concentration of myoglobin in the muscle.

Parvalbumins have also been detected in the brain and kidney.

Key words : Parvalbumin, Lungfish, fish muscle, Protopterus dolloi.

Introduction.

The bony fishes can be subdivided into two major groups, the so-called ray-finned fishes or actinopterygians and the fleshy-finned fishes or sarcopterygians. This latter stock is made itself of two subclasses of archaic fishes : the Dipnoi or lungfishes, the early specialization of which has restricted their subsequent evolution and the Crossopterygii which have given rise to two evolutionary trends, one leading to marine species with only one surviving specimen, the Coelacanth (*Latimeria chalumnae*) and the other to tetrapods and higher vertebrates [1]. The sarcopterygians occupy a key position in the evolutionary tree of early vertebrates and can be considered as very close to the ancestry of land animals. This position as well as the fact that many species of this group have kept most of the morphological characters of fossil animals could explain why, despite the difficulties encountered in the availability of fresh specimens, a great deal of efforts have been spent to elucidate their physiological and biochemical characteristics.

Various studies (for a review see [2]) have been devoted to the Coelacanth ; among them some reports are dealing with the aspect of molecular evolution such as the recent comprehensive study carried out on the parvalbumins of coelacanth muscles [3, 4, 5].

The actual surviving Dipnoi group two families : the Ceratodidae with one surviving australian species the Barramunda (*Neoceratodus forsteri*) and the Lepidosirenidae, gathering five species, one from South America (*Lepidosiren paradoa*) and four from Africa : *Protopterus annectens, P. aethiopicus, P. dolloi* roughly from central Africa and *P. amphibius* from western tropical Africa [6]. All have an elongated body, eel like, and paired fins of filamentous structure. The most remarkable anatomical characteristic of these Dipnoi are the existence of bilobed and elongated lungs lying dorsally. Gills are reduced and inadequate for respiration. These species are therefore highly adapted to drought and can survive several years in the absence of water by producing a water impermeable cocoon of dried slime surrounded by a thick layer of dessicated mud. During this period, called estivation, the fish does not ingest food or

Abbreviations :

Ethylenediaminetetra-acetic acid : EDTA.
Polyacrylamide gel electrophoresis : PAGE.
Trichloro acetic acid : TCA.

water nor excretes waste products except respiration gases. This particularity also explains why most of the physiological and biochemical studies concerning these fishes, since the pioneer work of Smith [7], have been mainly polarized on the metabolism of the estivating fish [8] as for example on problems like respiration [9-10] and nitrogen metabolism [11, 12]. Other works concern molecular evolution of proteins such as structural studies on the activation peptide of trypsinogen [13] and on the thyroglobulins [14].

The aim of our study is to add new informations about the relatedness which can be found at the protein level between a lungfish representative, *Protopterus dolloi*, its close cousin the coelacanth and the amphibians represented by frog. We have chosen a family of Ca^{2+} binding proteins, the parvalbumins largely distributed in the animal kingdom [15] which are present in high amount in the white muscles of lower vertebrates in which they seem to act as soluble relaxing factor [16-18].

The present paper is a rather general survey concerning the parvalbumins of the *Protopterus dolloi* ; it will be followed by other papers on the amino acid sequences of the lungfish parvalbumins which will be compared to the already published primary structures of the parvalbumins from frog [19] and from coelacanth [4, 5]. This hopefully will provide at the molecular level, new evidence of the phylogenetic relatedness of these species.

Material and Methods.

The lungfishes (*Protopterus dolloi*) were obtained alive from the Zaïre through the courtesy of Prof. J. Franssen. They were kept in fresh water at 20°C and fed with pieces of pig liver. The animals were anesthetized by addition of MS 222 to the water and the different muscles were carefully dissected and kept in a frozen state at —32°C.

Isolation of the parvalbumins.

They were extracted at 4°C from 250 g of minced white muscles with 1.5 vol of 10 mM Tris-HCl, 2 per cent glycerol (v/v), 10^{-3} M 2-mercaptoethanol, buffer pH 8.7. The mixture was homogeneized one minute in an omnimixer and then stirred one hour in the cold room. The supernatant of the centrifuged homogenate was fractionated with cold acetone.

The fraction precipitating between 55 and 80 per cent was collected, dissolved in 0.05 M NH_4HCO_3 and dialyzed overnight against the same solution. The clear solution was chromatographed on a Sephadex G 75 column (45 × 5 cm) in 0.05 M NH_4HCO_3. The parvalbumin fraction was concentrated by ultrafiltration through Amicon UM10 membranes and then dialyzed 4 h against 0.015 M HCl-piperazine buffer pH 5.7.

The final solution was chromatographed on a DEAE-cellulose column (Whatman DE32). Proteinic fractions were concentrated by ultrafiltration and desalted on a Biogel P_2 column in 0.05 M NH_4HCO_3.

Distribution of parvalbumins in various muscles and tissues.

A weighed amount of about 1g of the frozen material was grinded with 500 mg of sand in 1.5 ml of the extracting buffer. The homogenate was centrifuged at 25000 g and the supernatants were examined by polyacrylamide gel electrophoresis, double immunodiffusion and immunoelectrophoresis.

Analytical methods.

Starch gel electrophoreses were made according to Scopes [20].

Polyacrylamide electrophoreses were run in slab gel (12 × 19 cm) using 7.5 per cent polyacrylamide, 2M urea and 0.02 M Tris-0.12 M Glycine (pH 8.6). The gels were stained 15 min in the following mixture : 75 ml methanol, 186 ml H_2O, 30 g TCA and 0.1 per cent of Coomassie blue R250 and destained in an acetic acid : methanol : water 1:2:7 mixture. Alternatively the gels were stained by O-dianisidine [21], in order to detect hemoglobin and myoglobin and also by a specific creatine kinase test [20].

The amino acid composition of the performic acid oxydized proteins and the tryptic peptide maps were obtained as described previously [22] but in the case of enzymic digestion the proteins were first denatured by a 2 h treatment in 6 M Guanidinium chloride, 0.05 M EDTA followed by desalting on a Biogel P_2 column in 0.05 M NH_4HCO_3 and lyophilisation.

The presence of a blocked N-terminal amino acid residue was ascertained by a dansylation procedure [23]. The identification of the acetyl group was made by hydrolysis of the protein (0.3 µmol.) with p-toluene sulfonic acid [24] 12 h at 107°C followed by gas chromatography analysis of the liberated acid [25].

The C-terminal amino acid residues were identified by hydrazinolysis of about 0.2 µmol. of the performic acid oxydized proteins [26].

Ca^{2+} measurements were made using an atomic absorption spectrometer Perkin-Elmer Model 303. The isoelectric points of the proteins were determined by a 2 h isoelectrofocalisation on Ampholine PAG plate, pH 3.5-9, using an LKB power supply 2103 and a constant power of 30 W.

The ultra-violet spectra were recorded with a double beam Hitachi Perkin-Elmer 124. Extinction coefficients were obtained from the absorbance at 259 nm of solutions whose concentrations were measured by amino acid analyses taking into account the respective contents of each protein in arginine.

Immunochemical methods.

The antibodies were raised against the purified parvalbumins obtained as lyophylized powder.

Two rabbits per antigen were immunized according to an already described procedure [27]. The method consists to spill directly the lymphatic ganglions of the rear legs with 0.4 ml of 1 mg/ml antigen solution added to an equal volume of Freund's complete adju-

vant. The immunization is completed by three subcutaneous injections in the back of 0.2 ml of the mixture. Three other injections of 1 ml each were made at 15 days intervals. After this period ear bleedings of 3 ml of blood allow semi-quantitative estimation of the concentration of precipitating antibodies. Rabbits were then bled from the carotid artery. Antisera were stored in 2 ml vials frozen at —32°C. Quantitative precipitin reactions were performed according to a previous method [28]. Precipitates dissolved in 0.1 M NaOH were analyzed by ultraviolet absorption at 287 nm using A = 0.0103 µg N^{-1} ml^{-1} cm^{-1} [29]. The absorbance of the parvalbumin at this wavelength was neglected.

Sometimes it was necessary to adjust to the same range the concentration of precipitating antibodies present in the sera. This was made by isolation of the γ-G-immunoglobulins by Na_2SO_4 precipitation [30]. The precipitates were dissolved by dialysis against 0.05 M NH_4HCO_3 and the solutions were lyophylized.

Immunodiffusions and immunoelectrophoreses were carried out on microscopic glass slides in 1.5 per cent agarose gel in phosphate-citrate buffer pH 7.1.

Results and Discussion.

The elution profile of a fractionated extract of lungfish white muscle, on Sephadex G75 is shown in figure 1 A whereas figure 1 B gives the diagram of the chromatography of the parvalbumin mixture on DEAE-cellulose. Depending on the specimen investigated the relative proportions of the five main parvalbumins which could be isolated vary somewhat considerably especially at the level of peaks corresponding to components III a and

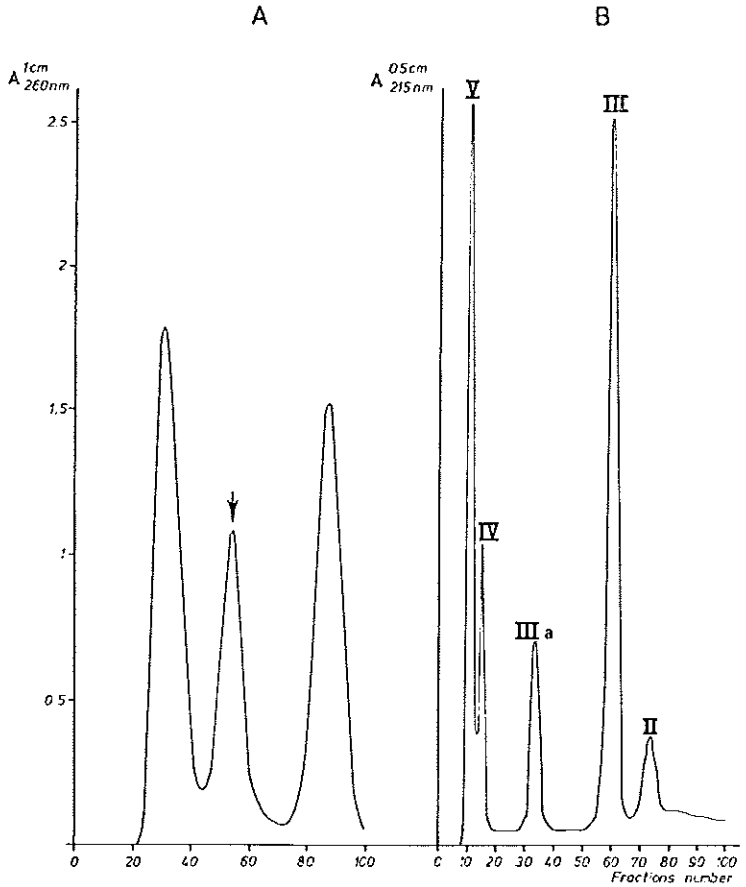

FIG. 1. — A. *Elution profile of the 55-80 per cent acetone fraction obtained from lungfish white muscle on a Sephadex G75 column* (45 × 5 cm) *equilibrated in 0.05 M* NH_4HCO_3. Fractions of 11 ml.

B. *Chromatography of the parvalbumins fraction obtained on Sephadex G75* (fig. 1 A) *on a DEAE-cellulose column* (30 × 2.5 cm) *equilibrated in 0.015 M HCl-piperazine*, pH 5.7. Eluting gradient was : 400 ml buffer — 400 ml 0.15 M NaCl buffer. Fractions of 7 ml.

II which can be much larger. Chromatographies of various unfractionated muscle extracts on AcA 54 column (LKB — Bromma — Sweden) reveal that parvalbumins occur in the lungfish white muscle at a mean concentration of 250 ± 20 mg per 100 g of fresh tissue ; this corresponds roughly to a 0.2 mM concentration in muscle.

The homogeneity of the isolated components was checked by starch gel electrophoresis (fig. 2). To obtain the component V and II in a relative pure state, it was necessary to rechromatograph the corresponding fractions of figure 1 B in other conditions : component V was purified by DEAE-cellulose chromatography in 0.015 M HCl-piperazine pH 7 using a salt gradient of 400 ml buffer-400 ml buffer, 0.15 M NaCl ; component II was rechromatographed on DEAE-cellulose in 0.05 M Tris-HCl pH 8 using a NaCl gradient of 0-0.15 M. Despite this, component V is often contaminated by denaturation products characterized by a faster rate of migration. It appears however chemically homogeneous by amino acid analysis (table I) and especially not contaminated by component IV.

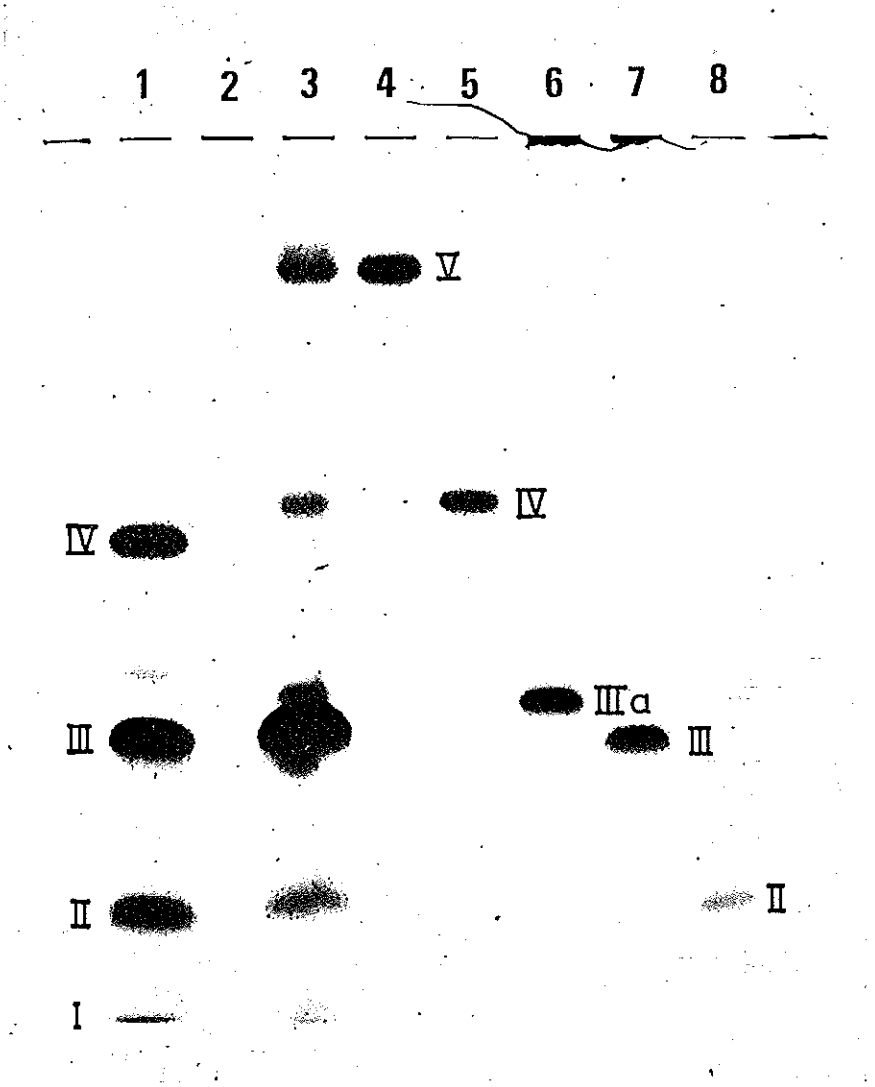

FIG. 2. — *Starch-gel electrophoresis pattern at pH 8.4 illustrating the process of purification of the parvalbumins from lungfish white muscle.* From left to right : slit 1 — parvalbumins from carp white muscle ; slit 3 — lungfish parvalbumins fraction from Sephadex G75 ; slit 4 to 8 — components V, IV, III a, III and II.

Chemical and physical properties.

The amino acid compositions of the five components are shown in table I. One can easily distinguish two families of parvalbumins on the basis of histidine and threonine contents : the first one contains components V, IV and III a and the second one components III and II.

rated per mole of protein. We conclude that the N-terminal amino acid residue is acetylated. The determination of the C-terminal amino acid residue confirms the existence of two families. The hydrazinolysis liberates leucine from components V, IV and IIIa with respective yields of 9, 26 and 16 per cent and serine from component III and II with 41 and 17 per cent of recovery.

TABLE I.

Amino acid composition of the performic acid oxydized parvalbumins from lungfish (Protopterus dolloi) *white skeletal muscle.*

The values listed are averages of six analyses obtained from 24, 48 and 72 h of hydrolysis.

Amido acid	Residues per mole of protein									
	Parvalbumin V		IV		III_A		III		II	
	Calculated	Assumed	Calculated	Assumed	Calculated	Assumed	Calculated	Assumed	Calculated	Assumed
Lys	15.8	16	14.1	14	13.3	13	9.5	10	10.0	10
His	2.0	2	1.8	2	1.9	2	0.0	0	0.0	0
Arg	2.1	2	1.9	2	2.1	2	1.9	2	2.4	2
Asx	14.6	15	15.9	16	15 3	15	16.1	16	15.1	15
Thr	1.6	2	1.7	2	1.1	1	4.9	5	5.0	5
Ser	11.1	11	9.7	10	7.6	8	10.9	11	10.0	10
Glx	11.1	11	12.3	12	11.9	12	12.4	12	14.0	14
Pro	0.0	0	0.0	0	0.0	0	0.0	0	1.6	2
Gly	6.7	7	8.1	8	6.4	6	9.9	10	9.2	9
Ala	8.8	9	10.8	11	10.8	11	11.1	11	11.7	12
Cys (a)	0.9	1	0.0	0	1.2	1	0.0	0	0.0	0
Val	7.0	7	7.3	7	6.2	6	3.9	4	4.7	5
Met (b)	2.7	3	0.0	0	1.9	2	0.0	0	0.0	0
Ile	5.6	6	7.9	8	7.9	8	6.1	6	5.0	5
Leu	7.6	8	7.8	8	7.4	7	10.2	10	9.9	10
Tyr	0.0	0	0.9	1	1.0	1	1.0	1	1.3	1
Phe	7.7	8	8.9	9	9.0	9	9.9	10	8 1	8
Trp (c)	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0
Total		108		110		104		108		108

(a) Determined as cysteic acid.
(b) Determined as methionine sulfone.
(c) Determined from U-V spectra.

In early experiments, the component II differed by the fact that no tyrosine and no proline residue was found. This could correspond to individual variations ; an alternative would be that two types of component II coexist in various relative amounts depending on the specimen examined so that the isolation of one or the other component could be favoured.

The dansyl-Edman method reveals that the terminal amino group is blocked. Hydrolysis and gas chromatography analyses indicated that a mean value of 0.94 ± 0.2 mole of acetic acid was libe-

The tryptic peptide maps also vary depending on the components investigated. Maps of component V, IV and IIIa are very similar ; they differ drastically from the maps of component II and III (fig. 3) which are somewhat different from each other despite an unquestionable relatedness. These two proteins are also extremely resistant to enzymic hydrolysis since apart from the usual denaturation performed in 6M guanidinium chloride and 0.05 M EDTA, it is necessary to heat the solution two minutes at 100°C just prior to the addition of 3 per cent of enzyme. A digestion time of at least 24 h is required.

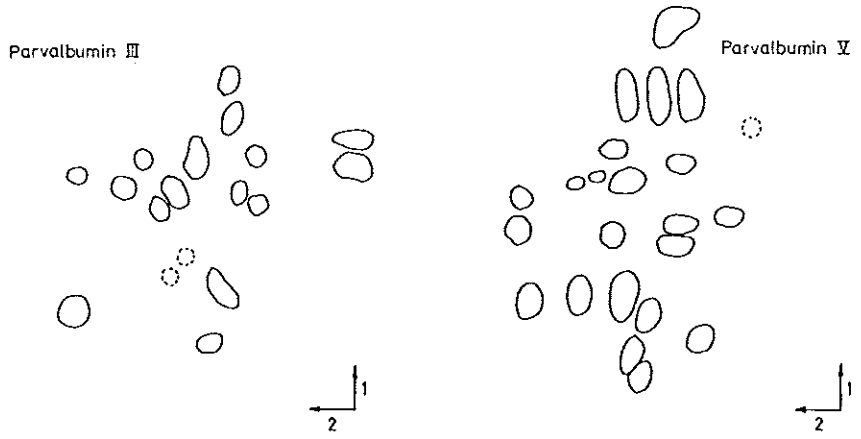

FIG. 3. — *Schematic representation of the tryptic peptide maps of the parvalbumins III and V obtained on cellulose thin layer plates.* First direction : chromatography in butanol/acetic acid/water (4:1:5, v/v). Second direction : electrophoresis at pH 3.7, 800 V for 50 min in pyridine/acetic acid/water (10:100:2890, v/v).

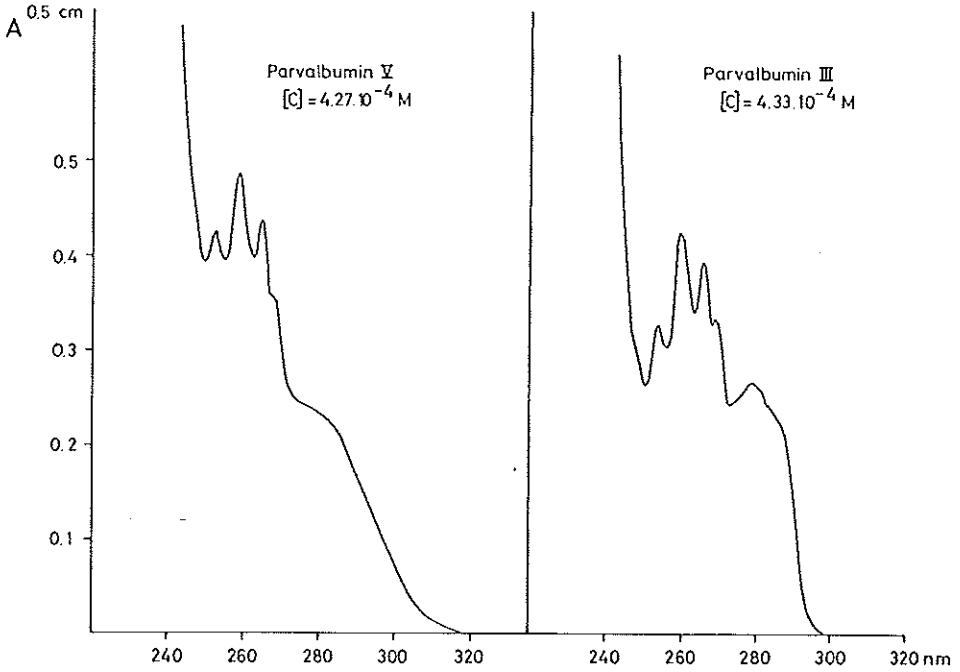

FIG. 4. — *Ultraviolet absorption spectra of the parvalbumins V and III of lungfish muscle*

The five components contain 2 ± 0.15 Ca^{2+} per mole of protein.

The isoelectric points are distributed on 2.6 pH units, some of them reach particularly high values which have never been mentioned so far for parvalbumins.

Parvalbumin	V	IV	IIIA	III	II
pI	6.6	6	5.4	4.3	4

The UV spectra are typical of this protein family. Molecular extinction coefficients at 259 nm determined from solutions, the concentrations of which were calculated by amino acid analyses are 2295, 2370, 2540 and 1986 mol^{-1} cm^{-1} for components V, IV, IIIa and III respectively. As an example the spectra of components V and III are illustrated in figure 4. The spectrum of compo-

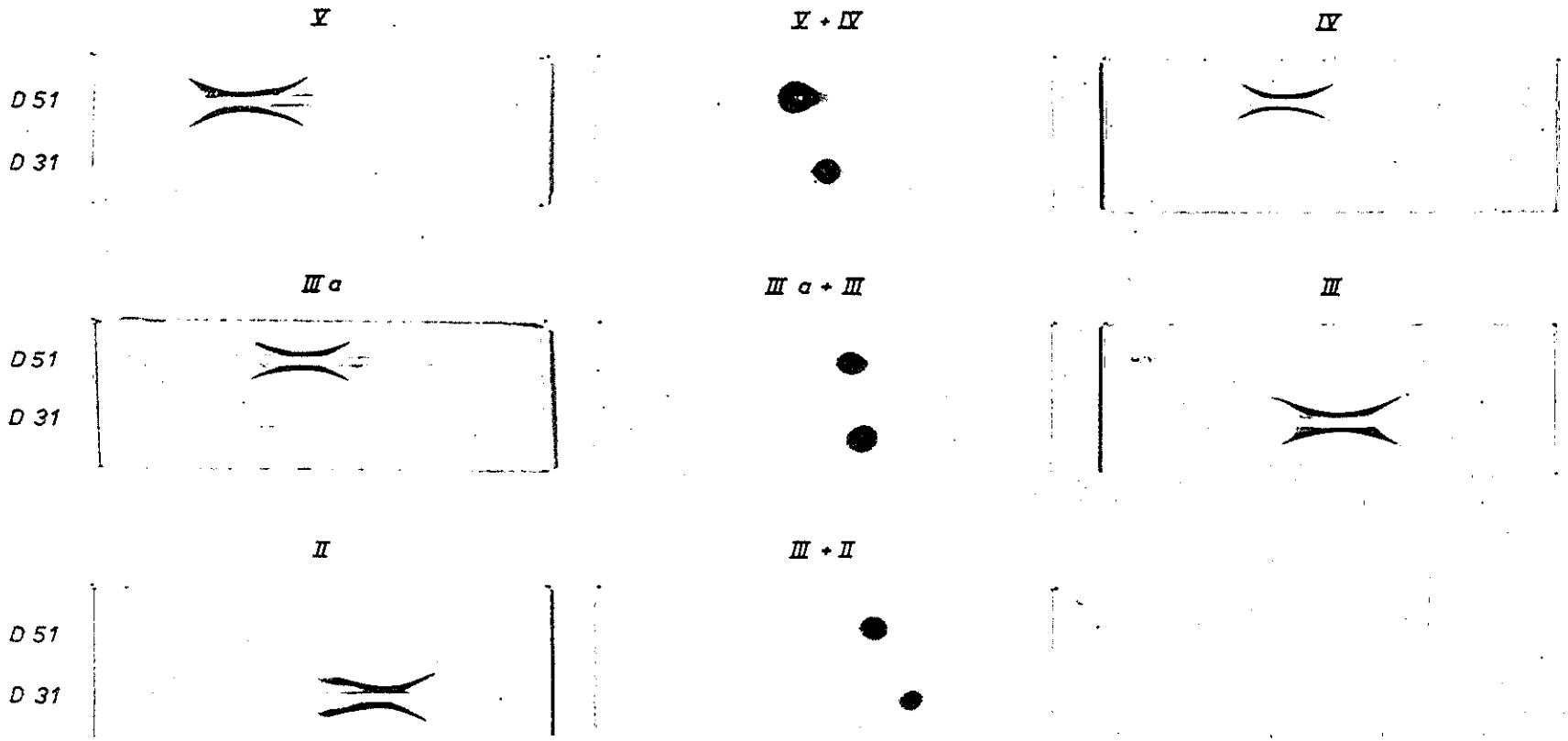

FIG. 5. — *Gel immunoelectrophoreses of the purified parvalbumins from the lungfish.* The upper troughs were filled with 100 µl of a solution (15 mg/ml) of γ-G-immunoglobulin isolated from serum D51 by Na_2SO_4 precipitation ; the lower troughs were filled with the same volume of a solution (4 mg/ml) of γ-G-immunoglobulins isolated from serum D31. The wells contain 2 µl of a parvalbumin solution (1.5 mg/ml) in phosphate buffer.

nent V shows a high residual absorption above 275 nm despite the fact that this component is devoid of tyrosine. Tentatives to remove it using denaturing agents like urea, guanidinium chloride or detergents were unsuccessful. This contaminating chromogen has already been noticed in the case of some parvalbumins by ourself and other [3].

Antigenic properties.

Antisera against the parvalbumin III were obtained from two rabbits and were named D31 and D32. The amount of precipitating antibodies was estimated from the precipitating curves assuming a composition of the precipitating complex corresponding to a maximum molecular ratio $\frac{Ab}{Ag} = 2$. This figure was obtained from the zone of excess antibodies determined by the analysis of supernatants by the ring tests [31]. The experimental values of this ratio obtained by extrapolation at zero antigen concentration were 2.34 and 2.30 for sera D31 and D32 respectively. D31 contained 6 mg/ml of precipitating antibodies (MW = 158000) and D32 8.4 mg/ml.

Only one rabbit reacted significantly against the parvalbumin V. Trial bleedings revealed a concentration of precipitating antibodies comparable to those obtained with the parvalbumin III. Unfortunately the carotid bleeding made ten days after the last trial gave a serum (D51) much poorer in precipitating antibodies which did not exceed 0.7 mg/ml.

The results of the immunoelectrophoresis of the five components against appropriate solutions of γ-G-immuno-globulins isolated by Na_2SO_4 precipitation from sera D31 and D51 are shown in figure 5. The heterogeneity of component V is demonstrated by the slight alteration in the regularity of the precipitating arc. All the other components show rather regular patterns. Components V, IV and IIIa do not give any precipitation with

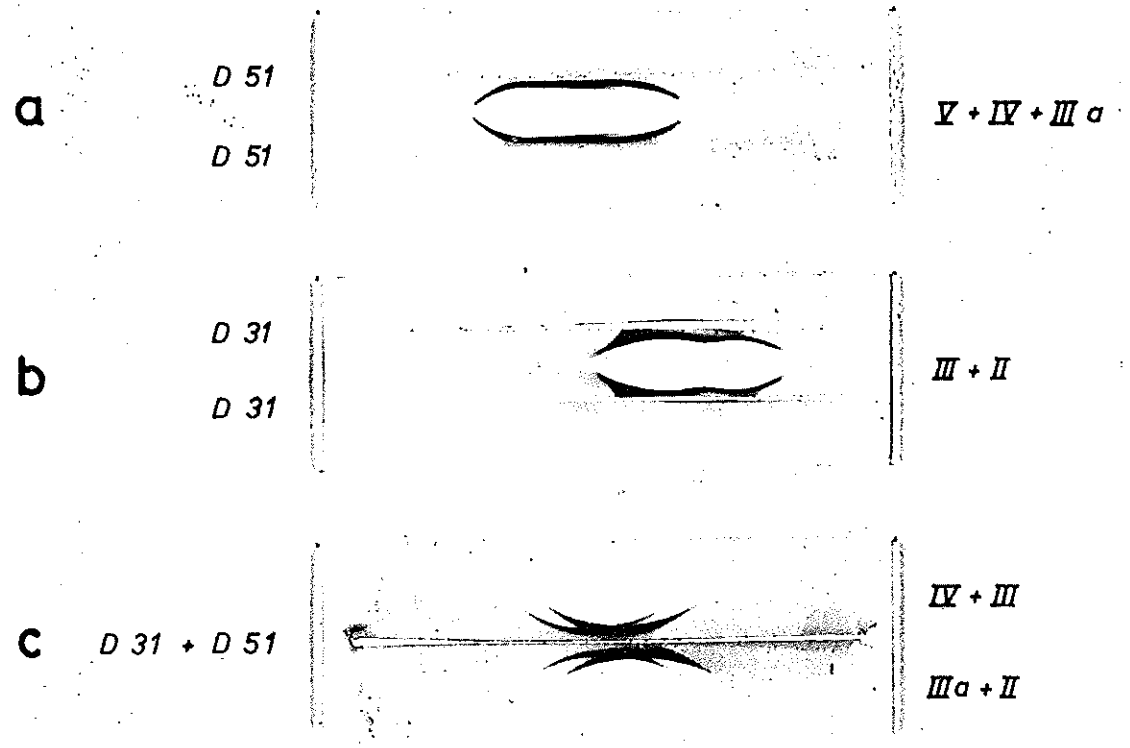

FIG. 6. — *Gel immunoelectrophoreses of synthetic parvalbumins mixture containing 1.5 mg/ml of each parvalbumin.* The troughs were filled with γ-G-immunoglobulins as in figure 5.

serum D31 ; on the other hand, components III and II react exclusively with serum D31. Examples of cross reactivities are shown in figure 6. As expected (fig. 6A) components V, IV and IIIa manifest immunoreaction of nearly complete identity towards serum D51 exception being for a very weak spur corresponding to component IV and projecting beyond the point of partial coalescence with component IIIa.

Component III and II seem to have strictly equivalent determinants towards serum D31 (fig. 6B).

No cross reactions are observed between on one hand component IV and III and on the other hand between component IIIa and II (fig. 6C).

All these results confirm the existence of two well definite parvalbumins families in the lungfish white muscle.

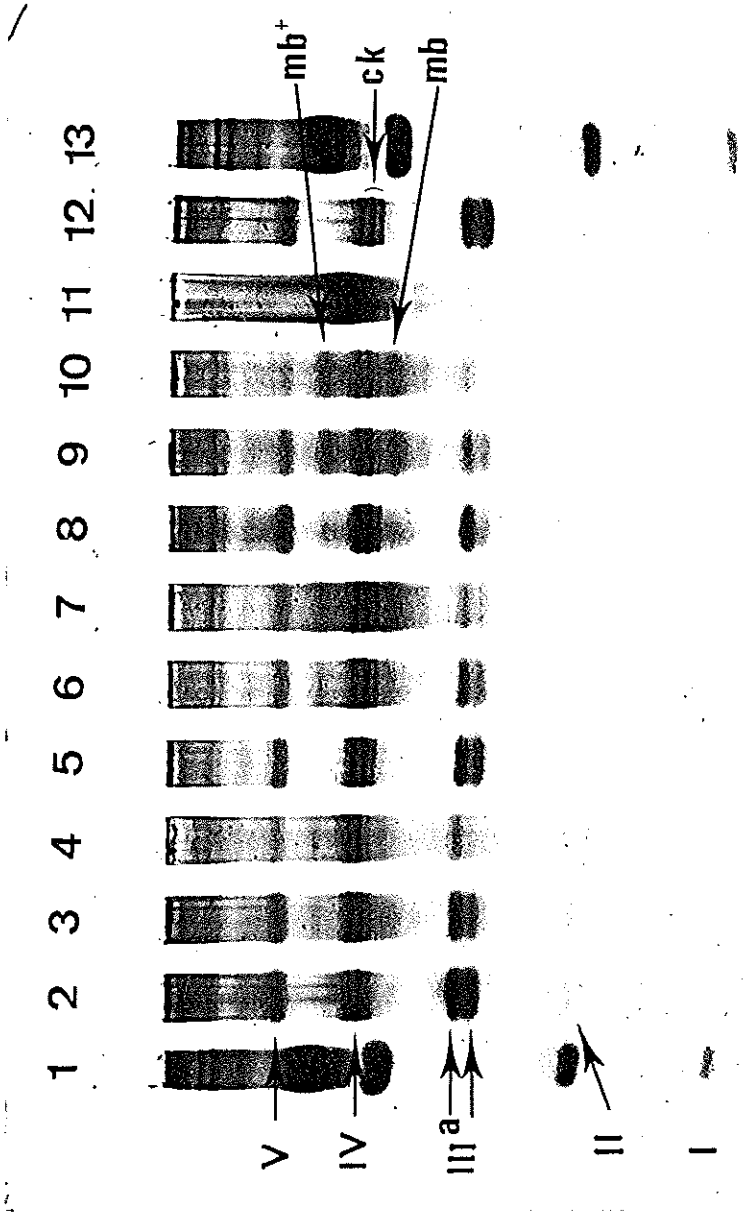

FIG. 7. — *PAGE patterns of various muscle extracts obtained at pH 8.6.* Total extracts.

1. Carp white muscle.
2. Lungfish white muscle.
3. Genohyoidian.
4. Anterior intermandibularis.
5. Sternohyoidian (pink).
6. Sternohyoidian (red part).
7. Protactor of cephalic rib.
8. Adductor inferior superficialis.
9. Lateral line.
10. Abductor and adductor of paired fins.
11. Ventricle.
12. Lungfish white muscle.
13. Carp white muscle.

Abbreviations :

mb+ : metmyoglobin.
Ck : creatine kinase.
mb : myoglobin.

Parvalbumins in various organs and muscles.

Various organs and muscles have been examined in order to study the distribution of parvalbumins. Ten muscles, brain, kidney and liver were analysed by polyacrylamide gel electrophoresis, by double immunodiffusion and by immunoelectrophoreses.

In the order of increasing red colour the muscles can be classified as follows :

trunk white muscle < sternohyoidian (inferior jaw) < genohyoidian (inferior jaw) <adductor inferior superficialis (jaw) < red part of sternohyoidian < intermandibularis (inferior jaw) < protactor of cephalic rib < lateral line < abductor and adductor of the paired posterior fins < ventricule. The PAGE patterns are shown in figure 7. All the muscles even heart contain parvalbumins but the patterns differ according to the muscle. White muscle along with geno-hyoidian and sternohyoidian, which are pink in colour, have the same assortments of parvalbumins, namely the five components, in comparable concentration. In the specimen investigated components III and IIIa occur in roughly similar amount. Component IV, not very abundant, is partly masked by three protein bands corresponding to creatine kinase. Metmyoglobin and myoglobin migrate on both side of this component.

The intermandibularis muscle (number 4) has a very weak amount of component III and its component II migrates faster than in the other muscles. The adductor inferior superficialis is very rich in components V and IIIa whereas ventricular muscle has a faint band migrating at the level of component IIIa. The double immunodiffusion tests confirm that all the extracts do contain parvalbumins giving precipitin reactions with sera D31 and D51. Even the extract from cardiac muscle gives a weak but quite discernible reaction especially with serum D51. The extracts from brain, kidney and liver do not show on PAGE visible traces of parvalbumins but double immunodiffusions carried out on these materials reveal in the brain extract the presence of parvalbumins reacting mainly with serum D51. Parvalbumins in the kidney were found more questionable.

Immunoelectrophoreses carried out on ventricle, brain, kidney and liver extracts show that parvalbumins V, IIIa and III are present in roughly similar amount in ventricle and brain extracts. A component migrating at the level of component III but giving only immunoprecipitation with serum D51 can also be localized in the kidney extract. No parvalbumin could be detected in liver extract.

Conclusions.

Five parvalbumins isotypes have been isolated from the white muscle of the lungfish. Their global concentration amounts to about 0.2 mM ; this is lower than the usual 0.4-0.5 mM concentration mentioned for other fish species [32, 35], much lower than the figure of about 1 mM reported for coelacanth white muscle [2] and roughly equivalent to parvalbumin concentration of frog white muscle [19, 22]. These five parvalbumins belong to two families differing from the points of view of amino acid composition, peptide map, C-terminal amino acid residue and immuno cross-reactivity. They probably originate from an early gene duplication. As the lungfish possess diploid cells of a very high DNA content [36], such a multiplicity of parvalbumin genes cannot be ascribed as in the Cyprinidae family to a polyploidization process [33, 37] but rather to a linear replication of some chromosomal regions followed by divergent evolution.

Comparison of various muscles shows on the other hand that, like in other fish species, the quantity of parvalbumins seems to be inversely related to the myoglobin content, the highest amount beeing found in white and the lowest in cardiac muscle [38].

The presence of parvalbumins in weak amount in fish cardiac muscle has already been pointed out earlier [39] and appears to be significant, their concentration in the heart is about ten times less than in the white muscle, in an antarctic myoglobin-hemoglobin free fish [40]. Mammalian heart on the contrary seems to be completely devoid of parvalbumins [35].

The significance of the inverse relation parvalbumin-myoglobin is uncertain. The only clue to the physiological role of these proteins appears so far to reside in their ability to play the role of a soluble relaxing factor [16-18]. On the basis of this interpretation the lower parvalbumin content in the red muscles could be explained by their slower contractile activity and by their higher content in mitochondria. This Ca^{2+}-trapping agent could be particularly efficient in cardiac muscle where mitochondria are particularly abundant [41] to lower the cytoplasmic Ca^{2+} concentration during the relaxation phase [42, 43].

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