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Sperm-Specific Basic Proteins in the Holocephalan Fish *Hydrolagus colliei* (Chondrichthyes, Chimaeriformes) and Comparison with Protamines from an Elasmobranch

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Abstract. Seven basic proteins can be isolated from sperm nuclei of the holocephalan ratfish *Hydrolagus colliei*. Two of these proteins (R3 and m0) are devoid of cysteine, whereas five of them (R1, R2, m1, m2, and m3) contain low levels of this amino acid residue. The proteins R1, R2, and R3 are major ones in the sperm nuclei of *H. colliei*, and they are analogous to basic proteins Z1, Z2, and Z3 (scylliorhinines) from the sperm of the elasmobranch *Scyliorhinus canicula*. However, taking into account the partial sequence of R3 protein and the number of cysteines in R1 and R2, these proteins do not seem to be homologous to the scylliorhinines. A comparison of sperm basic proteins between *H. colliei* (a holocephalan) and *S. canicula* (an elasmobranch) suggests a remarkable divergence of these proteins from a common ancestral pattern during the evolution of Chondrichthyes.

Introduction

The spermatozoan nucleus usually contains specific proteins that condense the DNA. In most animal spermatozoa, these proteins replace somatic histones. They show a great interspecific variability (Bloch, 1976; Kasinsky, 1989). The sperm-specific basic proteins (SBPs) of some bony fish (called "protamines," "monoprotamines," or "true protamines") have been very well studied at the levels of protein (Ando *et al.*, 1973; McKay *et al.*, 1986; Saperas *et al.*, 1993, a, b); gene (Dixon *et al.*, 1985); and genetic expression (Iatrou and Dixon, 1978; Hecht, 1989; Oliva and Dixon, 1991). The protamines of other vertebrates, particularly those from amphibians, reptiles, birds, and mammals, have often been considered to be molecules that are related evolutionarily to these protamines (Nakano *et al.*, 1976; Chiva *et al.*, 1987, 1989; Oliva and Dixon, 1991; Takamune *et al.*, 1991). Among the cartilaginous fishes, spermatogenesis in a few species has been studied based on cytochemical staining (Bols and Kasinsky, 1974, 1976) and electrophoresis (Oliveras *et al.*, 1990). However, at the level of biochemical characterization, knowledge of protamines in cartilaginous fish is based entirely on studies of a single species, *Scyliorhinus canicula* (Quero, 1984), the lesser spotted dogfish (also known as the small spotted catshark).

The observations on the changes of nuclear proteins that occur during the spermiogenesis of *S. canicula* show evident differences between this species and bony fish. Gusse and Chevaillier (1978, 1981) observed that two basic "intermediate" proteins, S1 and S2, appear in the early modifications of chromatin structure during spermiogenesis in *S. canicula*. These proteins partially substitute for histones (Chevaillier, 1991), although in more advanced stages they are in turn replaced by other basic proteins (protamines). These authors found four protamines (Z1, Z2, Z3, and S4) called "scylliorhinines" in the spermatozoan nucleus. One of these protamines (Z3) is a protein with 31 amino acid residues, a high percentage of arginine (64.5 mol%), and no cysteine (Sautière *et al.*, 1981; Gusse

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et al., 1983). This protein is organized in arginine clusters, and its primary structure is comparable with that of the protamines from bony fish. Z1, Z2, and S4 are cysteine-rich protamines and contain 50, 46, and 32 residues, respectively. The first two are rich in arginine, whereas S4 is rich in lysine (Gusse *et al.*, 1983). The sequences of these three molecules do not show a significant number of identities in the position of their residues (Sautière *et al.*, 1984; Martinage *et al.*, 1985; Chevaillier *et al.*, 1987); nor are they comparable to cysteine-rich protamines from mammals. In a general analysis from a comparison of the sequences of these proteins (Sautière *et al.*, 1984; Chevaillier *et al.*, 1987), these authors suggest two important points: (1) Z3 and bony fish protamines probably originated from the same ancestral DNA sequence, before the divergence between cartilaginous and bony fish occurred; (2) Z1, Z2, and S4 probably had an independent origin from different gene families. The studies of the sequence of intermediate protamines S1 and S2 also show that there is no relationship between their sequences and those from sperm protamines (Chauvière *et al.*, 1987, 1989).

In spite of this work, we cannot extend these results to chondrichthyan fish in general because *S. canicula* is the only species of cartilaginous fish that has been studied biochemically. In this work, we examine the sperm proteins of the ratfish (rabbitfish) *Hydrolagus colliei*. This species is a holocephalan (order Chimaeriformes). It was selected because the holocephalans diverged very early from the rest of the cartilaginous fish (Nelson, 1984). They are considered to be more primitive than elasmobranchs (Schaeffer, 1981). Consequently, a comparison between the protamines of *H. colliei* and *S. canicula* may provide valuable information about the common characteristics of these molecules in the early stages of the evolution of fish. We should take into account that the long period of separate evolution between these two groups could result in important differences between their sperm proteins.

Materials and Methods

Animals

H. colliei testes were obtained in April at the Friday Harbor Laboratories, San Juan Island, Washington, USA; in May at Comox, British Columbia, Canada; and in June at the Bamfield Marine Station, B.C., Canada.

Nuclei

Nuclei were prepared separately from whole testis, epididymis, and ampulla ductus deferentis after Gusse and Chevaillier (1978) as follows. The tissues were dissected on ice with scissors and homogenized for 30 s in a Sorvall Omnimixer in buffer A (20 m*M* Tris pH 7.5/0.15 *M* KCl/0.34 *M* sucrose) containing either 50 m*M* benzamidine chloride or 0.5 m*M* PMSF as a proteolytic inhibitor. The homogenate was filtered through four layers of cheesecloth and centrifuged at 4000 × *g* for 8 min. The resulting sediment (1 vol) was homogenized in 15 vol of buffer A containing proteolytic inhibitor and 0.1% (w/v) Triton X-100 in a Dounce hand homogenizer under the same conditions. The resulting homogenate was underlaid by 15 vol of buffer B (20 m*M* Tris pH 7.5/0.15 *M* KCl/1.4 *M* sucrose) containing the proteolytic inhibitor, and centrifuged for 20 min at 30,000 × *g*. The sediment was washed in distilled water and the nuclei (or chromatin) obtained again by centrifugation (4000 × *g*, 8 min). All the procedures were performed in the cold.

Proteins

Proteins were extracted from nuclei with 0.25 *N* HCl. In the cases that will be detailed below, nuclear sediments were reduced and alkylated before (or between) the extractions. For *H. colliei*, we applied the method used by Gusse *et al.* (1983) on *S. canicula* to allow the comparison of sperm protamines between both species. Nuclear sediments were reduced with 20 vol of buffer C (50 m*M* Tris, pH 8.8/10 m*M* DTT/2 m*M* disodium EDTA) in a 1-h incubation at 37°C under N_2 atmosphere. After centrifugation at 7700 × *g* for 10 min, the sediment was alkylated with 25 vol of 12.5 m*M* iodoacetamide in buffer C for 1 h at the same conditions and centrifuged.

For Figure 1, basic nuclear proteins were prepared from testicular zones. In this case, the micromethod of Louie and Dixon (1972) was used to isolate and extract sperm basic proteins from cell suspensions. We have utilized this method previously, as described in detail by Kasinsky *et al.* (1985).

Chromatography

Chromatographic separation of proteins by ion exchange columns was performed on CM-52 cellulose (Whatman). Proteins were dissolved in 50 m*M* acetate buffer (pH 6.0) containing 0.2 *M* NaCl (Chiva *et al.*, 1987, 1988). In some cases, the proteins extracted from sperm nuclei were reduced and alkylated again before chromatographic separation. Reduction was performed in buffer D (20 m*M* DTT/8 *M* urea/0.5 *M* NaCl) for 1 h at 37°C under N_2 atmosphere. Alkylation with 50 m*M* iodoacetamide in buffer D was done under the same conditions (Gusse *et al.*, 1983). Afterwards, the proteins were dialyzed against acetate buffer/NaCl and loaded onto the columns.

When necessary, fractions collected from CM-cellulose columns were applied on a 5 μm Spherisorb column and purified by reverse-phase high-performance liquid chromatography (HPLC). The column was equilibrated with 0.05% trifluoroacetic acid (TFA) and proteins separated with a gradient of acetonitrile in 0.05% TFA.

Amino acid analysis

Amino acid analyses were performed as described by Chiva and Mezquita (1983). These analyses were used to determine the molar compositions of proteins. The number of amino acid residues in each protein was inferred from the compositional values and from the electrophoretic mobility in acetic-urea polyacrylamide gels (Daban *et al.*, 1991).

In addition, we used the method described by Creighton (1980) and Hollecker (1989) to determine the integral number of cysteine residues of the molecules. This method has been applied to *H. colliei* protamines as well as to a commercial standard (BPTI, Boehringer Mannheim). The protein was denatured and reduced by incubating in 8 *M* urea/10 m*M* DTT/10 m*M* Tris-HCl pH 8.0/1 m*M* disodium EDTA for 30 min at 37°C. Afterward, aliquots of the sample were alkylated by adding 0.25 *M* iodoacetamide, 0.25 *M* iodoacetate, and varying ratios of iodoacetamide to iodoacetate, respectively. After 15 min at room temperature, the solutions were placed on ice and adequate volumes analyzed in polyacrylamide gels containing 8 *M* urea, following the low pH discontinuous system of Reisfeld *et al.* (1962).

N-terminal sequence

The amino-terminal sequence of ratfish protamine R3 was determined by automated Edman degradation with an Applied Biosystems 470A Protein Sequencer, and the phenylthiohydantoin derivatives of amino acid residues were analyzed on an Applied Biosystems 120A on-line HPLC system using a microbore C18 Brownlee column (2.1 × 220 mm) (Saperas *et al.*, 1993b).

Electrophoresis

The procedure of Panyim and Chalkley (1969) was used for routine electrophoretic analysis of proteins. The gels were stained with 0.25% (w/v) Coomassie blue in methanol/acetic acid/H_2O (5:1:1 by volume) and destained in the same mixture. Gels were 10 cm long, but sometimes 20-cm gels were used to improve resolution. To count the cysteine residues of the protein molecules, we used the methodology described by Reisfeld *et al.* (1962), but using gels containing 8 *M* urea.

Histology

In two specimens, midsagittal sections were dissected from concentric zones of ampullae in the testis (Stanley, 1963). Starting from the ampullogenic zone, a portion of each zone was fixed in 10% neutral-buffered formalin and used for cytochemical analyses (Bols and Kasinsky, 1974, 1976). The remainder of each zone was used to isolate and extract sperm basic proteins from nuclei by the micromethod of Louie and Dixon (1972).

Results

Nuclear proteins in testicular and sperm cells

For the electrophoretic analysis of basic nuclear proteins during the course of testicular spermatogenesis, one testis was dissected into concentric zones (A to E). A portion of each zone was examined cytochemically to establish the stages of spermiogenesis present in the tissue that had to be examined by electrophoresis. Spermiogenesis in the ratfish has been divided into seven stages on the basis of nuclear protein cytochemistry (Bols and Kasinsky, 1976). In Figure 1 left, zones A and B are equivalent to cytochemical stages 1 and 2 identified by Bols and Kasinsky (1976), and they contain round spermatids (A) and spermatids just beginning to undergo nuclear elongation (B). Zone C (stages 3–4) is heterogeneous and shows spermatids starting the process of spiralization, as well as those still in the midst of nuclear elongation. In zone D (stages 5–6), spermatids are completing spiralization, while in zone E (stage 7) the testicular sperm cells are organized into tightly packed cysts. None of these zones is completely homogeneous, but each zone (except for C) is enriched for cells at a particular stage of spermiogenesis.

The electrophoretic analysis of basic nuclear proteins from each of these zones is shown in Figure 1, right. This analysis reveals that somatic histones are the main proteins contained in nuclei from early spermiogenic stages, but in the most advanced stages, the spermatid nuclei contain a collection of proteins with higher mobility than histone H4. Three of these proteins appear to be major; they have been designated as R1, R2, R3. The proteins appearing in Figure 1 have been extracted from nuclei with 0.25 *N* HCl without previous reduction/alkylation of these nuclei. A subsequent reduction/alkylation and extraction of the chromatin did not solubilize any additional proteins.

Nuclei of sperm cells have been isolated separately from epididymis and the anterior and posterior region of the ampulla ductus deferentis. Figure 2 shows the proteins extracted from these nuclei with and without reducing conditions for chromatin. When nuclei are reduced and alkylated, a complex set of proteins can be solubilized with 0.25 *N* HCl extraction (Fig. 2, lanes b, c, d). The three major bands coincide with R1, R2, and R3 observed in spermiogenic testicular nuclei, but some minor bands also appear in the electrophoretic pattern (arrows in Fig. 2). When nuclei are extracted with 0.25 *N* HCl without previous reduction, only the protein R3 (Fig. 2, lane e) plus one minor band (named m0 in lane e) can be solubilized. Proteins R1 and R2 can be solubilized from sperm nuclei only after reduction of chromatin (Fig. 2, lane g). In this case, some minor bands also appear in the electrophoretic pattern (designated as m1, m2, and m3 in lane g). We have found these electrophoretic patterns to be highly reproducible with many sperm-nuclei preparations using different proteolytic inhibitors. We conclude

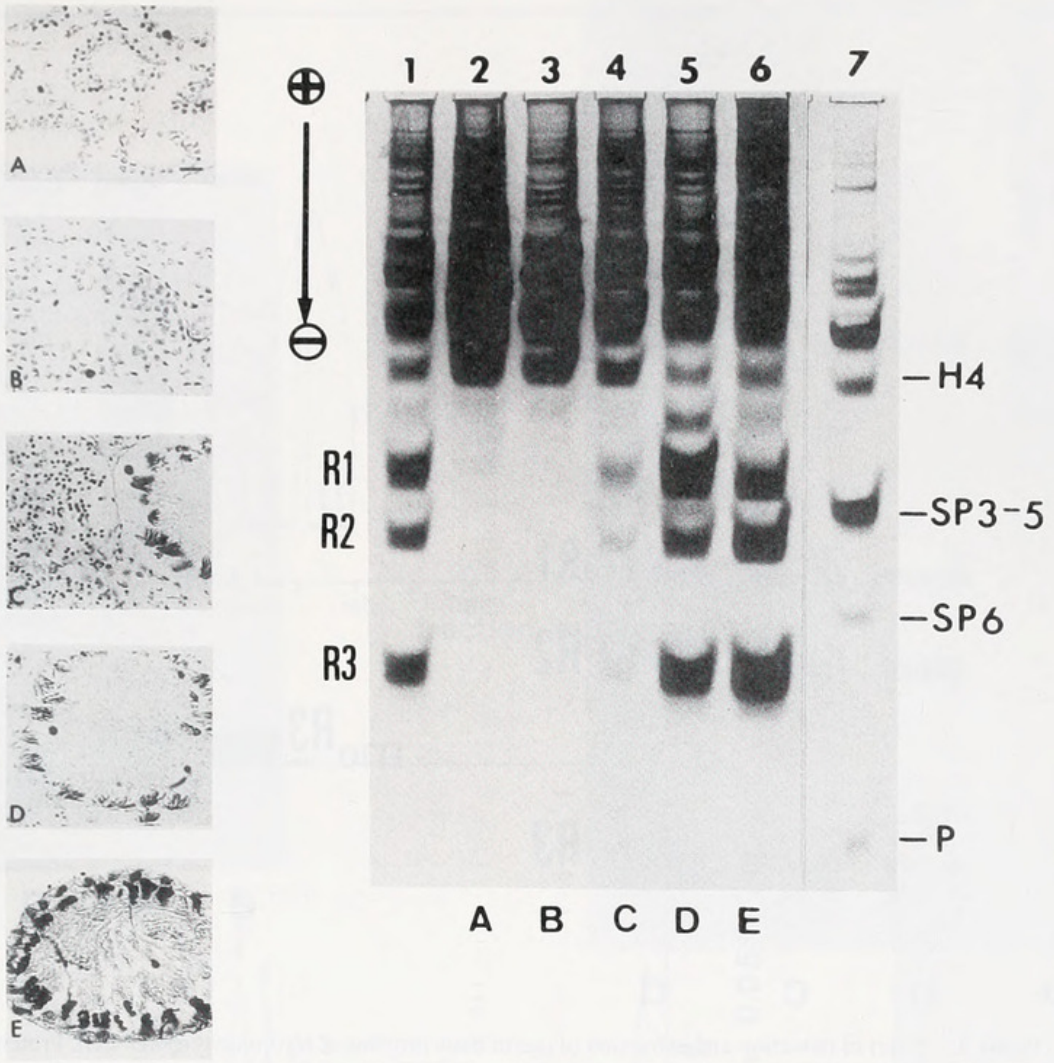

Figure 1. Left: Midsagittal sections of *Hydrolagus colliei* testis showing follicles containing different stages of spermiogenesis. Feulgen staining, zones A–D; hematoxylin-eosin staining, Zone E. Zones A and B, spermatids beginning the process of nuclear elongation; Zone C, mixture of spermatids undergoing nuclear elongation and spermatids beginning the process of spiralization; Zone D, spermatids undergoing spiralization; Zone E, sperm organized into tightly packed cysts. Scale (in A) denotes 10 μm for each zone. Right: Electrophoretic profiles of basic proteins extracted with 0.25 *N* HCl (without reduction) from dissected testicular zones. Lane 1, *H. colliei* whole testis; lanes 2–6, *H. colliei* testicular zones A–E, respectively; lane 7, standards of frog (*Xenopus laevis*) sperm basic proteins (histones and proteins SP 3–5, SP 6) and herring protamine (P). H4 = evolutionary conservative histone H4. Electrophoresis in this and subsequent figures is from top (+) to bottom (−).

that sperm-specific basic protamines of *H. colliei* consist of three major (R1, R2, and R3) and four minor (m0, m1, m2, and m3) proteins. Only two of them (R3 and m0) can be extracted from sperm nuclei without previous reduction. In addition, R1 and R2 reproducibly have a slightly lower mobility when extracted without reduction from testis (Fig. 2, lane f) than when extracted with reduction from epididymal sperm (Fig. 2, lane g).

Purification and analysis of proteins

We used sperm cells from epididymis, as well as anterior and posterior parts of the ampulla ductus deferentis, for the purification of ratfish nuclear sperm proteins. Nuclei were extracted directly with 0.25 *N* HCl, cleared by centrifugation; and reduced, alkylated, and re-extracted with 0.25 *N* HCl. The fractions containing proteins solubilized without reduction (R3 and m0) and proteins extracted after reduction (R1, R2, m1, m2, and m3) were precipitated with the addition of six volumes of cold acetone (−20°C overnight), rinsed with acetone, and dried.

The former fraction (R3 and m0) was placed on a CM-cellulose column and proteins were collected in two "peaks" (I and II, and Fig. 3A). Whereas peak II contained the purified protein R3 (Fig. 3, lane II), peak I contained a mixture of R3 and m0 (Fig. 3A, lane I). Proteins from this peak were separated by reverse-phase HPLC. In this system we obtained a series of peaks, some of them con-

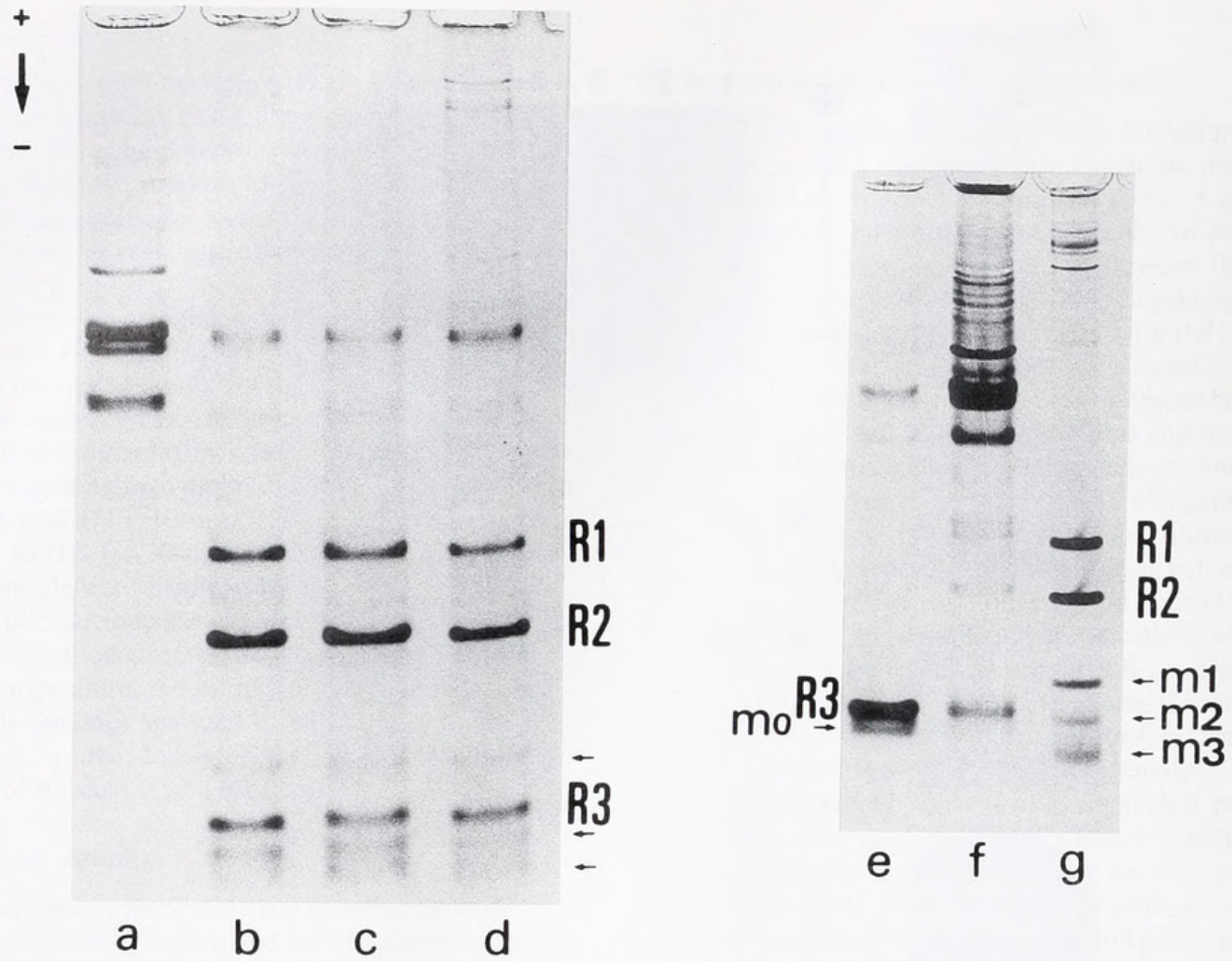

Figure 2. Effect of reduction and extraction of sperm basic proteins of *Hydrolagus colliei*. Left: Proteins extracted with 0.25 *N* HCl after reduction and alkylation of sperm nuclei. Lane b, sperm from epididymis; lane c, sperm from the anterior region of ampulla ductus deferentis; lane d, sperm from the posterior region of the ampulla ductus deferentis. Lane a shows a standard of histones from unripe testes of *H. colliei*. Arrows next to lane d indicate minor bands m1, m2, m3. Right: Proteins extracted with 0.25 *N* HCl without previous reduction. Lane e, epididymal sperm nuclei; lane f, testicular nuclei. Lane g shows the pattern when epididymal sperm basic proteins remaining in nuclei after removal of R3 and m0 (with 0.25 *N* HCl, lane e) are re-extracted with 0.25 *N* HCl after reduction/alkylation.

taining the purified proteins R3 and m0 (Fig. 3B). Proteins R3 and m0 from those columns were analyzed for their amino acid content. Compositional values are shown in Table I together with the amino acid composition of non-keratinous protamine Z3 from *S. canicula*. The amino acid content of *H. colliei* R3 is remarkably different from that of *S. canicula* Z3 (see Discussion).

An aliquot of the purified protein R3 was dialyzed extensively against 50 m*M* NH_4HCO_3 and lyophilized. For sequencing purposes, the protein was further purified by reverse-phase HPLC (see Materials and Methods). The 16 N-terminal amino acid sequence for protein R3 obtained by automated Edman degradation (Fig. 4) shows two heterogeneous clusters of basic amino acid residues (RRRH) and (KKKRK). The comparison of this part of the R3 molecule with the complete sequence of Z3 protamine of *S. canicula* supports the idea emerging from the amino acid analysis data that there is a large difference between ratfish R3 and dogfish Z3.

The proteins extracted with 0.25 *N* HCl after reduction of sperm nuclei were purified using the same types of chromatography as before. First we separated three fractions (I, II, and III in Fig. 5A) by ion exchange on CM-cellulose. Fraction II contained purified R1, and Fraction III contained R2 (Fig. 5C, lanes c, d). Fraction I contained the rest of R1 plus the minor proteins m1, m2, and m3 (not shown). Proteins from fraction I were purified in reverse-phase HPLC (Fig. 5, B and C) and analyzed compositionally. Table II shows their amino acid composition compared with scylliorhinines Z1, Z2, and S4. Although the compositional values are partially similar amongst the ratfish sperm basic proteins and scylliorhinines Z1 and Z2, this does not mean that they all belong to the same family of proteins; *i.e.*, ratfish and dogfish proteins need

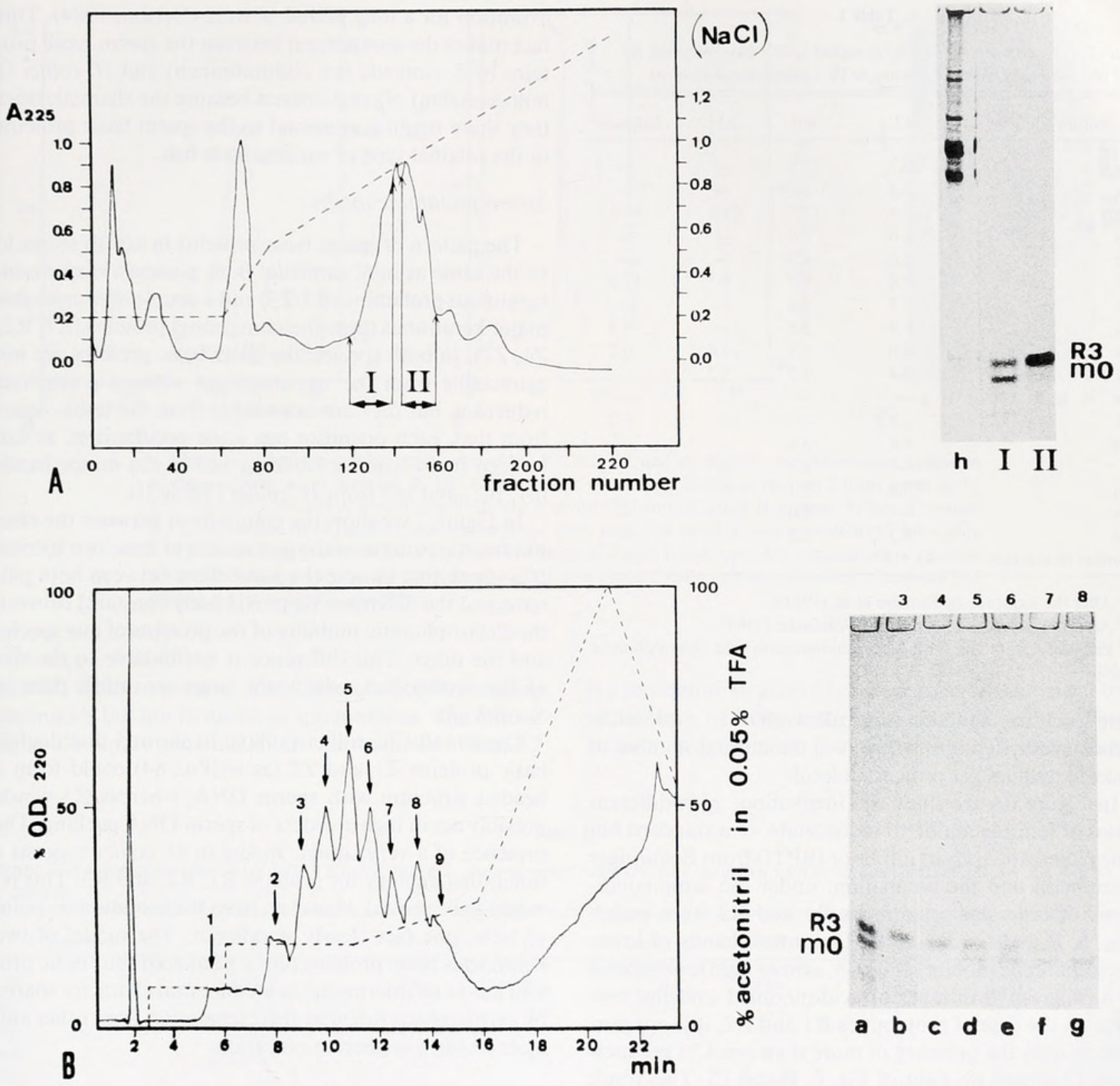

Figure 3. Chromatographic purification of R3 and m0 proteins. A. CM-cellulose chromatography of proteins extracted from sperm nuclei without previous reduction. Molarity of NaCl is shown on righthand vertical axis. Fraction II contains the purified protein R3 (see electrophoretic control on right). Lane h shows histones from unripe testes of *Hydrolagus colliei*. B. HPLC of protein contained in peak I from panel A. Fractions 3 and 4 contain R3 protein and fraction 8 contains m0 protein.

not be homologous. In fact, scylliorhinines Z1 and Z2 may not be homologous to each other. In spite of possessing comparable amino acid compositions, Z1 and Z2 do not have similar sequences (Chevaillier, 1991). The cysteine content is also significantly different: whereas scylliorhinines are very rich in this amino acid residue, protamines R1 and R2 from ratfish (and also the minor protamines m1, m2, and m3) have a poor content of cysteine.

To verify the low content of cysteine in ratfish sperm basic proteins, we applied the method described by Creighton (1980), which relies on the charge differences introduced by specific chemical modification of the amino acid. In Cys residues, the reaction of the thiol group with iodoacetic acid introduces a new acidic group, whereas reaction with neutral iodoacetamide does not change the net charge. Thus, the method consists of adding varying ratios of iodoacetamide to iodoacetate to portions of the protein to generate a com-

Table I

Amino acid composition (mol %) of ratfish sperm basic proteins R3 and m0 compared with Z3 protamine (S. canicula) *and salmine*

Amino acid	R3	m0	Z3[a]	Salmine[b]
Lys	20.7	23.8	—	—
His	4.3	5.6	—	—
Arg	27.6	23.7	64.5	65.6
Asx	4.6	1.0	—	—
Thr	2.6	4.9	—	—
Ser	7.4	9.3	9.7	12.5
Glx	5.7	7.4	—	—
Pro	6.9	3.5	—	9.4
Gly	4.0	3.5	19.4	6.2
Ala	3.4	2.7	3.2	—
Cys	—	—	—	—
Val	1.9	—	—	6.2
Met	1.6	3.4	—	—
Ile	—	1.8	—	—
Leu	3.8	6.1	—	—
Tyr	—	2.0	3.2	—
Phe	5.3	1.2	—	—
Number of residues	45 ± 4[c]		31	32

[a] After the sequence by Sautière *et al.* (1981).

[b] After the sequence by Ando and Watanabe (1969).

[c] Estimated from the amino acid composition and electrophoretic mobility.

plete spectrum of protein molecules with 0, 1, ...*N* acidic carboxymethyl groups, where *N* is the integral number of cysteine residues per protein molecule.

In Figure 6A we show the incubation, with different ratios of iodoacetamide to iodoacetate, of a standard bovine pancreatic trypsin inhibitor (BPTI) from Boehringer Mannheim and the incubation, under the same conditions, of testicular protamines R1 and R2 from ratfish (Fig. 6, B and C). In the BPTI control, bands of lower electrophoretic mobility (Fig. 6A, arrows right) correspond to a progressive number of acid-modified cysteine residues. In the case of protamines R1 and R2, it is not possible to infer the presence of more than one Cys per molecule (2 arrows on right of Fig. 6, B and C). This result agrees with the previous one obtained by amino acid analysis.

Discussion

The holocephalans diverged very early from the other cartilaginous fish and therefore have followed a separate evolution for a long period of time (Nelson, 1984). This fact makes the comparison between the sperm basic proteins of *S. canicula* (an elasmobranch) and *H. colliei* (a holocephalan) of great interest because the characteristics they share might correspond to the sperm basic proteins in the original type of cartilaginous fish.

Sperm protamine model

The pattern of sperm basic proteins in ratfish seems to be the same as in *S. canicula.* Both possess a major nonkeratinous protamine (R3/Z3) and a couple of comparable major keratinous (cysteine-containing) proteins (R1, R2/Z1, Z2). In both species, the latter basic proteins are not extractable from the spermatozoan without a previous reduction, but they are extractable from the testis. Apart from that, each organism has some peculiarities, as can be seen in S4 from *S. canicula* and in the minor bands m1, m2, and m3 from *H. colliei* (Table II).

In Figure 7 we show the comparison between the electrophoretic patterns of the protamines of these two species. It is interesting to note the parallelism between both patterns and the difference (approximately constant) between the electrophoretic mobility of the proteins of one species and the other. This difference is attributable to the size of the protamines, which are larger in ratfish than in *S. canicula.*

Gusse and Chevaillier (1980a, b) showed that dogfish basic proteins Z1 and Z2 (as well as S4) could form a beaded structure with sperm DNA, whereas Z3 would possibly act in higher orders of sperm DNA packing. The presence of a very similar model in *H. colliei* suggests a functional analogy for proteins R1, R2, and R3. This remains to be proved. However, from the evolutionary point of view, one fact clearly stands out: The model of two keratinous basic proteins plus a nonkeratinous basic protein has to be interpreted as an ancestral character shared by cartilaginous fish after their separation from other animals in early vertebrate evolution.

Characteristics of the molecules

In a series of studies on the scylliorhinines, Gusse, Chevaillier, Sautière, and colleagues (Sautière *et al.,* 1981, 1984; Martinage *et al.,* 1985; Chevaillier *et al.,* 1987, 1989) sequenced the four protamines of *S. canicula* (Z1, Z2, Z3, and S4), as well as the two intermediate proteins (S1

	1	5	10	15	20	25	30
(a)	A R R R H S M K K K R K S V R R						
(b)	A R S R S R R S Y G R G R R R G G R R R R R R R R R R G G R						

Figure 4. N-terminal sequence of (a) R3 sperm protein from *Hydrolagus colliei* compared with the sequence of (b) Z3 from *S. canicula* (Sautière *et al.,* 1981).

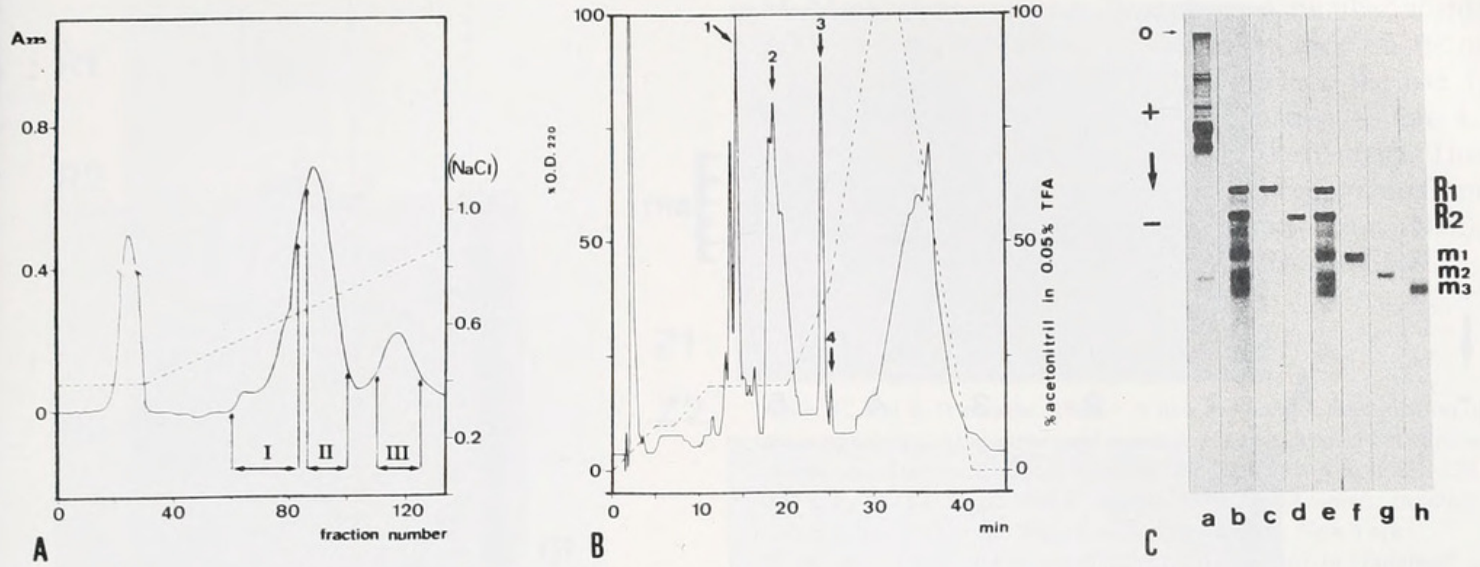

Figure 5. Chromatographic purification of R1, R2, m1, m2, and m3 proteins. A. CM-cellulose separation of the complete set of proteins; B. HPLC purification of proteins eluted in fraction I from panel A; C. Electrophoretic control of proteins purified: lane c, protein R1 from fraction II of panel A; lane d, protein R2 from fraction III of panel A; lane f, protein m1 from peak 3 of panel B; lane g, protein m2 from peak 4 of panel B; lane h, protein m3 from peak 1 of panel B. Lanes b and e are the complete set of proteins after 0.25 *N* HCl extraction and the reduction/alkylation process. Lane a shows a standard of histones and protein R3 from *Hydrolagus colliei*.

and S2) that are present in the nuclei of differentiating spermatids but not in nuclei of spermatozoa. The following evolutionary generalizations can be made. First, Z3 is a protamine structurally very similar to the typical true protamines of bony fish (Sautière *et al.*, 1981). These authors suggest that "scylliorhinine Z3 and teleost

Table II

Amino acid composition (mol %) of ratfish R1, R2, m1, m2, and m3 sperm basic proteins compared with keratinous protamines Z1, Z2, and S4 of S. canicula

Amino acid	R1	R2	m1	m2	m3	Z1[a]	Z2[b]	S4[c]
Lys	12.2	9.9	20.1	21.0	15.6	16.0	13.0	43.7
His	6.9	1.2	6.5	7.7	5.4	4.0	6.5	—
Arg	30.1	39.3	35.4	30.0	29.2	32.0	37.0	21.9
Asx	4.3	1.7	1.5	2.8	1.2	4.0	—	—
Thr	2.8	4.6	—	1.8	4.8	4.0	2.2	—
Ser	4.0	9.7	10.5	4.0	8.7	4.0	4.3	—
Glx	6.0	1.5	6.7	4.9	5.1	2.0	2.2	—
Pro	6.9	3.8	—	4.8	—	6.0	2.2	6.2
Gly	6.4	21.1	7.6	5.2	7.7	2.0	2.2	3.1
Ala	2.7	3.8	—	3.6	9.3	2.0	10.9	9.4
Cys[d]	1.2	1.4	2.0	1.3	1.9	8.0	8.7	12.5
Val	3.5	—	5.1	2.3	7.8	4.0	4.3	3.1
Met	1.2	—	—	1.9	—	—	2.2	—
Ile	3.9	1.7	—	1.1	—	—	2.2	—
Leu	4.5	1.8	2.3	3.3	3.3	8.0	2.2	—
Tyr	2.2	—	1.8	1.0	—	2.0	—	—
Phe	1.2	2.3	—	3.3	—	2.0	—	—
Number of residues:	79 ± 4[e]	66 ± 2[e]				50	46	32

[a] After the sequence by Chevaillier *et al.* (1987).
[b] After the sequence by Martinage *et al.* (1985).
[c] After the sequence by Sautière *et al.* (1984).
[d] Cys determined as carboxymethyl-cysteine.
[e] Estimated from the amino acid composition and electrophoretic mobility.

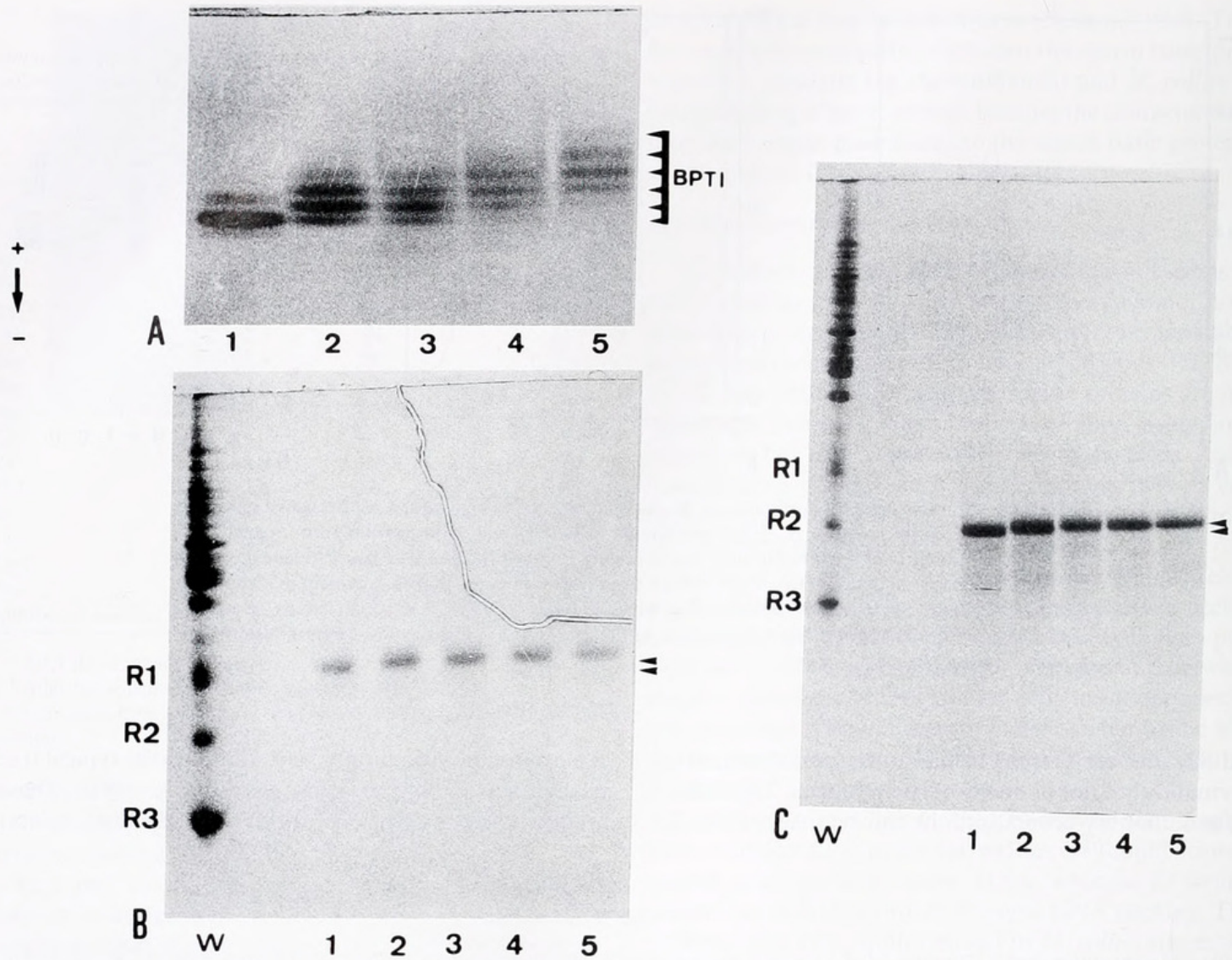

Figure 6. Determination of the number of cysteine residues according to the method of Creighton (1980). A. Standard of BPTI (Boehringer Mannheim); B. R1 from *Hydrolagus colliei*; C. R2 from *H. colliei*. Reaction with iodacetamide (1); iodoacetamide/iodoacetate in ratios of 1:1 (2), 1:3 (3), and 1:9 (4); and with iodoacetate (5). W = whole basic protein pattern from *H. colliei* testis.

protamines originated very probably from the same ancestral DNA sequence before the divergence of Chondrichthyes and Osteichthyes during the Devonian period" (Chevaillier *et al.*, 1987). Second, there are very few identities in the sequences of protamines Z1, Z2, and S4. Possibly, these basic proteins have evolved neither from the same single ancestral polypeptide nor from the same gene family. Third, the intermediate basic proteins S1 and S2 are not homologous to the sperm proteins. These data mean that the sperm (and spermiogenic) nuclear proteins from *S. canicula* are coded by a series of genes that express coordinately during spermiogenesis but do not possess a close evolutionary relationship.

The comparison of ratfish protamines with those from *S. canicula* brings out yet a greater variability in this system of molecules. Ratfish R3 protamine differs greatly from *S. canicula* Z3 protamine (Table I). R3 contains a relatively great diversity of amino acid residues (14 different types), contrasted with only five types of residues that constitute Z3. In addition, ratfish is relatively poor in arginine (27.6 mol % *versus* 64.5 mol % in dogfish), and its N-terminal sequence shows the presence of clusters of heterogeneous basic residues (RRRH, KKKRK) that are not present in Z3, nor in the protamines of bony fish. The minor protamine m0 presents compositional characteristics similar to R3 (Table I).

The comparison between the amino acid composition of R1 and R2 and the scylliorhinines Z1 and Z2 (Table II) shows that, globally, all these proteins possess a comparable composition. However, this similarity does not necessarily mean that there is a homology between them. Two points are inconsistent with such a homology: (1) scylliorhinines Z1 and Z2 are compositionally similar but their sequences present hardly any identities; (2) there are important deviations in the compositions of R1 and R2 with respect to Z1 and Z2, as can be seen by the larger

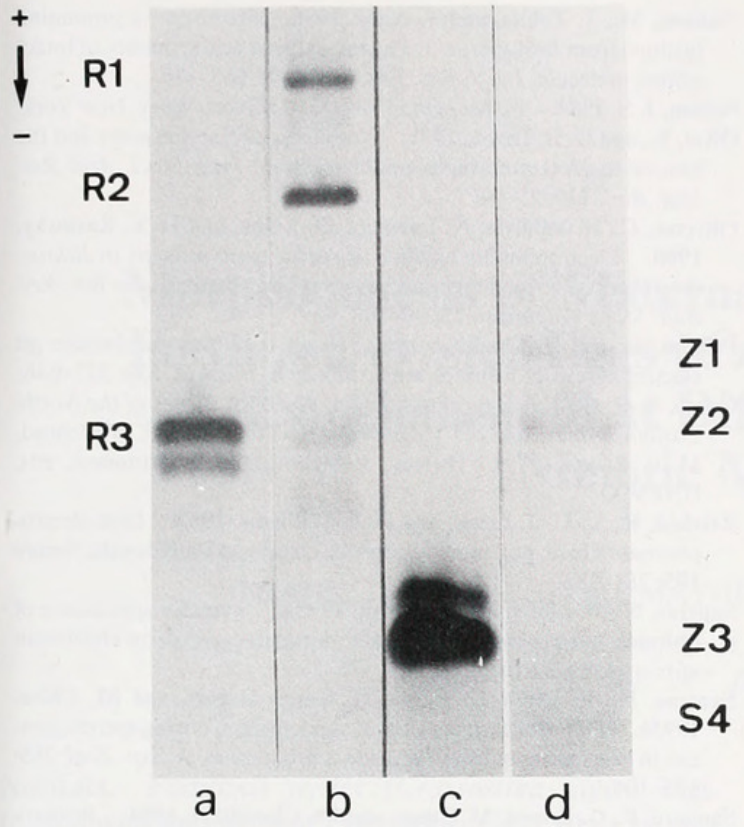

Figure 7. Comparison of electrophoretic patterns of sperm basic proteins from *Hydrolagus colliei* (lanes a, b) and *S. canicula* (lanes c, d): proteins without cysteine (lanes a and c) and cysteine-containing proteins (lanes b and d).

proportion of glycine in ratfish R2 (21.1 mol %) and the presence of a very low percentage of cysteine in both R1 and R2.

The results presented here show a high variability among chondrichthyan protamines, possibly due to an origin from a different pool of genes or, alternatively, to great divergence while retaining the ancestral pattern of two keratinous protamines and one nonkeratinous protamine. Finally, the great difference between proteins R3 from ratfish and Z3 from dogfish calls into question the interpretation (Sautière *et al.*, 1981) that nonkeratinous protamines from Chondricthyes and typical protamines from bony fish have the same common ancestor.

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