Calcified Ectodermal Collagens of Shark Tooth Enamel and Teleost Scale

Abstract. *Amino acid analysis of protein from the enamel of shark teeth and from teleost scales shows the presence of collagens which can be classified chemically as ectodermal. This finding, together with results from a histological examination of the development of these tissues, constitutes strong evidence that both proteins are derived from the ectoderm, like the enamel of higher vertebrates. Since both are calcified, calcification cannot be a specific property of collagens of mesodermal origin alone.*

Shark teeth and the closely related dermal denticles are covered by a glistening, hard layer of enamel. The precise homology of these tissues with enamel from higher vertebrates has been the subject of continued controversy. Utilizing similar histological data, previous workers have not been able to agree whether the enamel is derived from the ectoderm or the mesoderm. The hypothesis of ectodermal derivation implies the active participation of the inner enamel epithelium of the shark enamel organ in amelogenesis (*1*). For the hypothesis of mesodermal derivation, the enamel matrix must be regarded as the product of mesodermal pulpal cells alone; the resulting tissue has been designated as mesodermal enamel (*2*), vitrodentine (*3*), or durodentin (*4*). If the mesodermal origin is correct, the enamel of higher vertebrates must be considered to be of relatively recent origin, having developed first in tetrapods. If the ectodermal origin is correct, enamel must share a phylogenesis with other calcified skeletal tissues which appeared in the earliest vertebrates.

A related problem is presented by teleost scales. According to current opinion, there is a structural difference between the inner fibrillar layer (or plate) and the outer calcified layer, sometimes termed "hyalodentine." Calcification by hydroxyapatite occurs in the upper half of the fibrillar plate as well as in the outer layer. The composition of fish scales is variable. The protein content of the scale varies between species from about 40 to 85 percent (*5*). About three-quarters of this protein is similar to or identical with fish bone collagen (*6*). It can be extracted by autoclaving or heating the scale with dilute hydrochloric acid; the residue thus obtained has been called "ichthylepedin" (*5*). Recent evidence indicates that this substance may also be a collagen (*7*).

The precise origin of the cells responsible for the formation of the scale tissues remains a matter of some debate. While most workers consider the entire scale to be derived from the mesoderm, substantial data suggest active ectodermal participation (*8*). Accordingly, it may be that the shark enamel organ and the teleost scale pocket are homologous structures. If this is true one might reasonably expect to find ectodermally derived tissue components in both shark enamel and in the outer layer of the teleost scale.

We have investigated the problems of the phylogenesis of shark enamel and of teleost scales, utilizing two independent approaches; in one, the tissues were studied by histological techniques, including autoradiography, and in the other, we studied the amino acid chemistry of the organic matrix. Here we report primarily on the amino acid composition of these two tissues (*9*).

Ichthylepedin was isolated from carp scales by the procedure of Green and Tower (*5*). Enamel protein was obtained from a group of large shark teeth, apparently representing several species, purchased commercially. The basal "bone of attachment" was removed and the teeth were ground to pass a 50-mesh screen. The enamel and dentin were separated by differential flotation in pure bromoform, according to a modified procedure of Manley and Hodge (*10*). The fractionation was repeated three times, and the fractions were washed with acetone and then with carbon tetrachloride. The dry dentin and enamel were decalcified in 5 percent ethylenediaminetetraacetic acid, *p*H 7.5, at 5°C for 7 days. The organic residue was washed thoroughly with water and dried over calcium chloride. Hydrolysates were prepared and amino acid analyses were performed, as described previously, in an automatic amino acid analyzer (*6*).

The interpretation of the results (Table 1) is aided by the well-established observation that collagens can be clearly divided into two groups (*11, 12*). One of these groups includes the majority of collagens found in vertebrate species, whether derived from hard or soft tissues. These collagens are all mesodermal in origin and show certain species- and tissue-specific differences in amino acid composition, but they represent a closely related group of proteins of relatively recent origin and apparently appeared first in the earliest vertebrates. In addition to the collagen from shark dentin analyzed as part of this study, previous analyses of shark skin (*13*) and

Table 1. Amino acid composition of some vertebrate collagens. Results expressed as residues of amino acid per 1000 total residues.

Amino acid	Shark			Carp		
	Enamel	Dentin	Skin (*17*)*	Scale ichthylepedin	Scale gelatin (*6*)	Skin (*6*)
Hydroxyproline	62	71	60	87	82	73
Aspartic acid	107	57	43	46	48	47
Threonine	27	25	23	34	25	27
Serine	96	51	61	55	43	43
Glutamic acid	73	72	68	80	69	74
Proline	80	102	106	103	117	124
Glycine	248	321	338	263	326	317
Alanine	63	107	106	71	119	120
Valine	38	26	25	31	18	19
Methionine	10	14	18	29	14	12
Isoleucine	28	20	15	32	11	12
Leucine	36	26	25	35	22	25
Tyrosine	29	6.2	3.3	25	3.3	3.2
Phenylalanine	18	14	13	20	14	14
Hydroxylysine	17	12	5.5	15	7.1	4.5
Lysine	27	19	27	16	25	27
Histidine	10	7.3	13	30	5.2	4.5
Arginine	33	51	51	24	52	53
Tryptophan		0	0		0	0
Cystine (half)	0	0	0	4.2	0	0
Amide N†	93	44	36	68	30	26

* References shown in parentheses. † Not included in total residues.

carp skin and scale gelatin (*6*) are included in Table 1 as representatives of this group.

The second group contains all of the known invertebrate collagens. These are ectodermal in origin, insofar as this term can be applied to invertebrates. They have an ancient origin, extending at least as far back as the sponges. In contrast to the collagens of mesodermal origin, they are distinguished by an amino acid chemistry of great diversity. Although they fulfill the chemical requirements for classification as collagens (containing about one-third glycine, as well as hydroxyproline and hydroxylysine), their chemistry otherwise shows no regular pattern. On the basis of both ectodermal derivation and a chemistry unlike that of the mesodermal collagens, several vertebrate collagens must be included in this group, among them "ovokeratin" of the skate egg, vitrosin which is a fibrillar element in the vitreous body, and the protein of lens capsule (*12*).

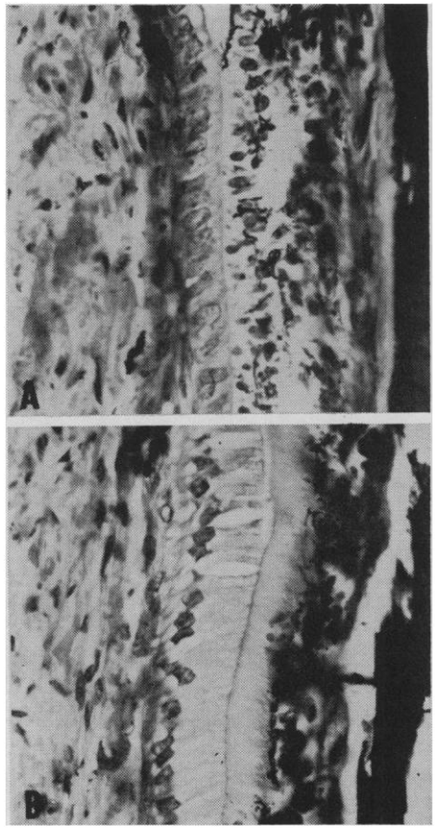

Fig. 1. Amelogenesis in the dorsal spine of a fetal spiney dogfish (*Squalus acanthias*). (*A*) An immature area of the enamel organ. The inner enamel epithelium is the vertical column of rectangular cells. Fish lack any stratum intermedium or stellate reticulum. The outer enamel epithelium is often difficult to see, as in this section. Pulpal cells and pigment granules can be seen. (*B*) An actively functioning area of the same specimen. The cytodifferentiation of the inner enamel epithelium is typical of active vertebrate amelogenesis. The enamel matrix is shown in this decalcified section to the right of the ameloblast. It is important to note that this tissue is formed centrifugally, external to the pulpal pigment, confirming the earlier report of Lison (*1*).

As shown in Table 1, the proteins of shark enamel and carp scale ichthylepedin fall unequivocably among the second group. Although both are undoubtedly impure, it is highly unlikely that the presence of impurities can explain the large differences between these proteins and the mesodermal collagens from the same species. For example, the low content of alanine and arginine in shark enamel protein, and of alanine, lysine, and arginine in ichthylepedin, could in each case arise only if 40 to 50 percent of protein impurities containing none of these amino acids were present. Similarly, the considerably higher content of aspartic acid, serine, valine, tyrosine, hydroxylysine, and lysine in shark enamel protein and of methionine, isoleucine, tyrosine, hydroxylysine, and histidine in ichthylepedin, relative to the mesodermal collagens, could be explained only by the presence of large amounts of impurities of very unusual composition. Therefore these data constitute strong evidence supporting the hypothesis of ectodermal derivation of the protein of the enamel of shark teeth and of the ichthylepedin in teleost scale.

The proteins of shark and pig embryo enamel (*14*) are distinctly different. The latter cannot be classified as a collagen because of its low glycine content. However, it is probably a related protein since it contains hydroxylysine and large amounts of proline. The difference is seemingly related to the divergent ontogenetic processes involved in their development.

The position taken by the proponents of a mesodermal origin of shark enamel can probably be explained also by a consideration of odontogenesis in the shark in particular, and in fish in general. Unlike odontogenesis in tetrapods, the formation of the organic matrix of dentin does not precede the formation of the matrix of enamel. In all fish, the inner enamel epithelium of the enamel organ undergoes cytodifferentiation very reminiscent of tetrapod amelogenesis, while the enamel matrix is deposited centrifugally, prior to dentinogenesis (Fig. 1). The possibility that the matrix of the enamel might be of mixed origin was first suggested by Tomes (*14*). Kvam (*2*), though a strong advocate of the mesodermal hypothesis, has also suggested that a part of the enamel matrix is derived from the inner enamel epithelium. Our results demonstrate that not only is this true but that probably most of the protein matrix is ectodermal (*15*). Autoradiographic data support this statement (*10*).

The data presented here suggest an additional conclusion of significance to the problem of the role of protein matrices in calcification. The calcification of collagen must have been an important step in the evolution of vertebrates from invertebrates (*16*). For example, there are no known examples of calcified collagens among invertebrates. Therefore bone and other hard tissue formation in vertebrates might be associated causally with the development of mesodermal collagens, for both events were concurrent. However, the finding of two examples of ectodermal, invertebrate-like collagens in vertebrates which are calcified makes this hypothesis untenable. Hard tissues are apparently not uniquely dependent on collagens of mesodermal origin; their phylogenesis is presumably related to other, as yet unknown, factors.

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Aminoacyl Position in Aminoacyl sRNA

Abstract. *Chromatographic systems capable of resolving the 2′- and 3′-isomers of valyladenosine and* N*-acetylvalyladenosine are used to show that valyladenosine isolated from valyl-sRNA is the equilibrium mixture of 2′- and 3′-acyl isomers by the time of chromatography. Acyl migration in* N*-acetylvalyladenosine is shown to be a rapid, base-catalyzed reaction with a rate 40,000 times the rate of hydrolysis under equivalent conditions. Calculations indicate that the pool of free aminoacyl sRNA exists in vivo as the equilibrium mixture of the 2′- and 3′-aminoacyl isomers.*

The discovery that the aminoacyl sRNA is bound in an ester linkage (*1*) to the 2′- or 3′-hydroxyl group of the terminal adenosine residue leads to the problem of determining whether the ester linkage is 2′- or 3′- in the aminoacyl sRNA pool in vivo. The position of the aminoacyl group has been the subject of some speculation (*2*), and three chemical methods (*3–6*) have been devised to determine the acyl position in aminoacyl adenosine released from aminoacyl sRNA by pancreatic ribonuclease. McLaughlin and Ingram (*4*, *7*) and Wolfenden, Rammler, and Lipmann (*6*) found that the distribution of the 2′- and 3′-isomers was the same for the aminoacyl adenosines isolated from sRNA and for those prepared synthetically. In the absence of suitable standard compounds (such as a pure 2′- or a pure 3′-aminoacyl adenosine) it is difficult to tell whether acyl migration to the equilibrium distribution occurred before or during the chemical determination of the aminoacyl position, or at both times. Furthermore, if acyl migration between the 2′- and the 3′- hydroxyl groups is rapid under the conditions of the chemical determination of the acyl position, then the blocking reagent itself, by preferential reaction with either the 2′- or 3′-hydroxyl, may result in an apparent shift in the equilibrium distribution in favor of the aminoacyl ester of the hydroxyl group which is less reactive towards the blocking agent.

To avoid these difficulties in the chemical methods of determining acyl position, we developed a series of chromatographic systems based on buffered ammonium sulfate solutions capable of separating the 2′- and 3′-isomers of several 2′ (and 3′-)-*O*-valyladenosine derivatives. Figure 1 shows the separations observed with thin-layer cellulose (*8*) chromatography of 2′ (and 3′-)-*O*-valyladenosine and 2′ (and 3′-)-*O*-(*N*-acetylvalyl)-adenosine. The separation of the 2′- and 3′-valyl-isomers is not complete, and in all solvent systems tested the 2′-isomer streaks into the 3′-isomer. Elution of the material in the spots as marked in Fig. 1 indicates about 85 percent of the 3′-isomer and 15 percent of the 2′-isomer, but because of streaking this may not be the true equilibrium value. These numbers are to be compared with our results by the phosphorylation method (*4*) which gave a value of 67 percent for the 3′-isomer of valyl-adenosine. On the other hand, the chromatographic separation of the 2′- (and 3′-)-*O*-(N-acetylvalyl)-adenosine is complete; the equilibrium distribution is 75 percent of the 3′-isomer. Furthermore, studies on 2′ (and 3′-)-*O*-valyladenosine-5′-phosphate and 2′ (and 3′-)-*O*-(*N*-acetylvalyl)-adenosine-5′-phosphate suggest that the normal equilibrium distribution and aminoacyl groups in sRNA under aqueous conditions around neutrality is likewise about 3 to 1 in favor of the 3′-isomer (*7*).

Valyl-C^{14}-sRNA (specific activity 205 mc/mmole) was prepared enzymatically and the valyl-C^{14}-adenosine fragment produced by ribonuclease treatment was isolated by electrophoresis. The initial incubation to form aminoacyl sRNA had been carried out at *p*H 8.45 at 37°C for 15 minutes. A Sephadex column (G-50, *p*H 5.5, 2°C, 45 minutes) was used to remove the excess valine-C^{14}; the aminoacyl sRNA was then incubated with pancreatic ribonuclease at *p*H 5.5 for 10 minutes at 37°C. The solution was chilled and adjusted to *p*H 3.0 with acetic acid; the precipitate was removed, and the supernatant was submitted to paper electrophoresis at *p*H 3.0 for 3 hours at 10°C with synthetic valyladenosine as a standard. The band for synthetic valyladenosine was located on the damp paper by ultraviolet light; the band was cut out, and the material was eluted with 0.01*M* formic acid at 2°C. The eluate was concentrated by rotary evaporation and applied to a thin-layer cellulose plate for chromatography. The result, 86 percent of the valyladenosine as the 3′-isomer and 14 percent as the 2′-isomer, indicated that valyladenosine as isolated had the equilibrium distribution of aminoacyl groups. Calculations indicate that for base-catalyzed migration our isolation system should cause over 100-fold less acyl migration than does the isolation system which Zachau and co-workers (*5*, *9*) had developed to prevent acyl migration. For acid- or water-catalyzed migration the situation is much the same. However, the calculations also indicate that most of the base-catalyzed acyl migration occurs during the initial incubation rather than

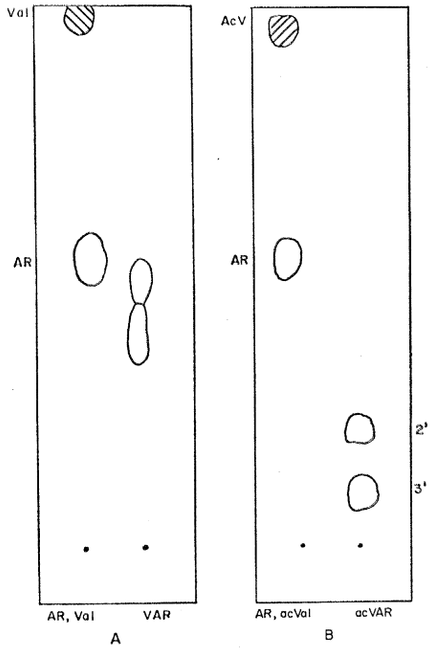

Fig. 1. Thin-layer cellulose chromatogram of 2′ (and 3′-)-*O*-valyladenosine (VAR) and 2′ (and 3′-)-*O*-(*N*-acetylvalyl)-adenosine (acVAR). Chromatographic solvent *A*: Saturated ammonium sulfate adjusted to *p*H 1.0 with concentrated sulfuric acid. Chromatographic solvent *B*: A mixture of saturated ammonium sulfate and 1*M* ammonium formate *p*H 3.2 (80 : 20). Abbreviations: AR, adenosine; val, valine; acVAL, *N*-acetylvaline. The 2′- and 3′-isomers are assigned on the basis the equilibrium distribution observed chromatographically and by phosphorylation.