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Compact Exocrine Pancreas of Ammocoetes of the Southern Hemisphere Lamprey *Mordacia mordax* Contains a Trypsin Inhibitor: Putative Evolutionary Considerations

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ABSTRACT The localization of exocrine pancreatic (zymogen) cells in the epithelium of the anterior intestine of the larvae of holarctic lampreys (Petromyzontidae) is considered the most primitive arrangement of these cells in vertebrates (Barrington, '72). In contrast, the zymogen cells are located in prominent forward-projecting diverticular extensions of the anterior intestine in larvae of the two monogeneric southern hemisphere lamprey families (Geotriidae and Mordaciidae). During the present study, tryptic inhibitory activity was not found in the two diverticula of *Geotria australis,* whereas a trypsin inhibitor of the Kazal type was found in the diverticulum of *Mordacia mordax,* eluting at the same chromatographic position as the inhibitor of rat pancreas. It is suggested that a trypsin inhibitor is not required in *G. australis,* because its wide duct-like diverticula would facilitate a relatively steady dispersal of trypsin into the anterior intestine. In contrast, a trypsin inhibitor would be of value to *M. mordax* since the highly folded epithelial lining and narrow central canal of its diverticulum would presumably lead to some trypsin being retained in the diverticulum. Such a protective mechanism against unbridled proteolysis would be complemented by the relatively very low level of trypsin activity found in the diverticulum of *M. mordax.* Consideration of our data in the context of the proposed scheme for the phylogeny of lamprey taxa suggests that a trypsin inhibitor has been evolved in the larval exocrine pancreatic tissue of one of the lamprey families, i.e., Mordaciidae, independent of that developed in the exocrine pancreas of gnathostomes.

The Agnatha (jawless fishes) are represented in the contemporary fauna only by the hagfishes and lampreys (Hardisty, '82). The living lampreys, which have an antitropical distribution, are separated into three families (Potter, '80a). All of the numerous northern hemisphere species are placed in the Petromyzontidae, whereas the four southern hemisphere species are divided into either the Mordaciidae or Geotriidae, both of which are monogeneric. Comparisons of a number of characters suggest that *Geotria* and *Mordacia* evolved separately from stocks similar to that now represented by the northern hemisphere genus *Ichthyomyzon* (Hubbs and Potter, '71; Potter and Hilliard, '87). In Australia, these two genera are each represented by a single anadromous parasitic species, namely *Geotria australis* and *Mordacia mordax* (Potter et al., '86).

The larval (ammocoete) phase in the life cycle of all species of lampreys is spent in fresh water and usually lasts for more than 3 years (Potter, '80b). Ammocoetes spend most of their time burrowed in the soft substrata of streams and rivers, feeding mainly on the overlying detritus and diatoms (Moore and Mallatt, '80). The enzymes responsible for digesting the food are produced by cells that are assumed to be homologous with the zymogen cells of the exocrine pancreas of higher vertebrates (Youson, '81; Hilliard and Potter, '88). However, the distribution of these cells differs markedly among the three lamprey families. Thus, while they are found in the anterior intestine of the Petromyzontidae, they are located mainly in a large left and small right diverticulum in the Geotriidae, and in a single large left diverticulum in the Mordaciidae (Hilliard and Potter, '88; Bartels and Potter, '95). In each of the latter two families, the diverticula, which have been regarded as constituting a protopancreas

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(Strahan and Maclean, '69), originate as forward extensions of the alimentary canal at the junction of the esophagus and anterior intestine. However, the internal epithelial lining of the diverticula of *Geotria* is not folded, whereas that of *Mordacia* is highly folded (Bartels and Potter, '95). As a consequence, each of the tube-like diverticula of *Geotria* has a large lumen, with a wide opening into the anterior intestine, whereas the diverticulum of *Mordacia* contains narrow spaces between the internal folds and a narrow canal running throughout its length (Bartels and Potter, '95).

Amylolytic, lipolytic, and chymotryptic activity have recently been shown to be present in the diverticula of ammocoetes of the two southern hemisphere lamprey families and in the anterior intestine of ammocoetes of the holarctic species *Lampetra richardsoni* (Cake et al., '92). However, while tryptic activity was likewise recorded in the corresponding locations in larval *Geotria* and *Lampetra,* such activity was negligible in the diverticulum of ammocoetes of *Mordacia.* This result was surprising since trypsin is the only known activator of chymotrypsinogen, and the chymotrypsinogen of *Mordacia* was activated by exogenous trypsin. One possible explanation is that a trypsin inhibitor is present in the diverticulum of *Mordacia* (Cake et al., '92). The presence of such an inhibitor in this structure would help protect the cellular lining of its highly folded internal walls from its own proteolytic secretions.

The present study was undertaken to determine whether a trypsin inhibitor is present in the diverticulum of the ammocoetes of *Mordacia*, as is the case in the complex pancreas of higher vertebrates (Rinderknecht, '86). The diverticula of larval *Geotria* were also analyzed to test the hypothesis that it would not contain a trypsin inhibitor, because trypsin would be likely to pass far more readily from the wide lumen of this structure into the anterior intestine. Further experiments were also performed in order to determine whether tryptic activity was present in the diverticulum of larval *Mordacia.*

MATERIALS AND METHODS

Chemicals

N-benzoyl-L-tyrosine ethyl ester (BTEE), bovine α-chymotrypsin (EC 3.4.21.1), bovine enteropeptidase (EC 3.4.21.9), bovine trypsin (EC 3.4.21.4), p-tosyl-L-arginine methyl ester (TAME), and tris (hydroxymethyl) aminomethane (Tris) were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride ($CaCl_2$) was supplied by Univar Ajax Chemicals (Sydney, Australia). Aprotinin came from Boehringer Mannheim GmbH (Mannheim, Germany), and BioGel P 30 from Bio-Rad Laboratories (Hercules, CA). Rat pancreatic juice was kindly provided by Associate Professor R. Morgan, University of Western Australia, Nedlands, Western Australia.

Preparation of tissue extracts

Ammocoetes of *Geotria australis* and *Mordacia mordax* were collected by electrofishing from rivers in southwestern and southeastern Australia, respectively. They were maintained in aquaria supplied with water and a natural substrate containing food, comprising diatoms and detritus, and subjected to analyses within a month of capture.

The ammocoetes selected for experiments ranged from circa 50–95 mm in length and would thus typically have been between 1 and 4 years old (Potter and Hilliard, '86). They were anesthetized in benzocaine and killed by decapitation. Each diverticulum was cut off just above its junction with the anterior intestine. Diverticula were then pooled, weighed to 0.1 mg, and homogenized in 10 vol of chilled 1.0 mM HCl containing 0.145 M NaCl, pH 3, using a tight-fitting teflon-glass homogenizer. The homogenate was centrifuged at 210 kPa (165,000 *g* max) at 4°C in a Beckman Airfuge for 12 min, and the supernatant collected and kept on ice until required.

Because the molecular weights of trypsinogen, i.e., 24,000 (Walsh, '70), and pancreatic trypsin inhibitor, i.e., 6,155 (Burck, '70), are very different, they were separated using molecular sieve chromatography. The column was standardized using standard bovine trypsin. Pancreatic juice from rat was subjected to chromatographic analysis to determine the position where trypsin and the trypsin inhibitor eluted. Due to the limited amount of tissue available, a 12 ml BioGel P 30 column (1.6 × 6 cm), eluted with 1.0 mM HCl containing 0.145 M NaCl, pH 3, was used for lamprey cytosolic extracts. The column was fitted with a pump and fraction collector, which delivered 265 μl fractions at a rate of 5 ml hr^{-1}. All elutions were conducted at 4°C.

Enzyme assays

In order to activate trypsinogen, aliquots of the chromatographic fractions from rat and lamprey extracts were exposed to enteropeptidase (175 μg ml^{-1}) in Tris-HCl buffer (28.75

mM), pH 8.1, containing $CaCl_2$ (7.2 mM). They were then incubated at 20°C for 180 and 45 min, respectively. Trypsin (EC 3.4.21.4) was subsequently assayed using the method of Hummel ('59). An extinction coefficient of 540 M^{-1} cm^{-1} is assumed (Rick, '74), and tryptic activity in the eluted fractions is expressed as μmol TAME hydrolyzed min^{-1} g^{-1} wet weight of tissue. A linear relationship was shown to exist between tryptic activity and the amount of trypsin, within the range 0–2.5 μg. Lamprey eluates were diluted so that they fell within this linear range.

Aliquots of chromatographic fractions from lamprey diverticular extracts were also exposed to trypsin (100 μg ml^{-1}) in Tris-HCl buffer (30 mM), pH 7.8, containing $CaCl_2$ (37.5 mM), and incubated at 20°C for 120 min in order to activate chymotrypsinogen. Subsequently, they were assayed for chymotryptic activity using BTEE as substrate, as described by Hummel ('59). An extinction coefficient of 964 M^{-1}·cm^{-1} is assumed and chymotryptic activity expressed as μmol BTEE hydrolyzed min^{-1} g^{-1} wet weight of tissue.

Assay of trypsin and chymotrypsin inhibitor

The reduction of the tryptic hydrolysis of TAME was used as a quantitative assay for trypsin inhibitor. Inhibitory activity was determined by incubation of 100 μl aliquots of the chromatographic fractions with 100 μl of purified bovine trypsin (50 μg ml^{-1}) for 10 min at 20°C and then assaying 100 μl of the mixture for trypsin activity using the procedure of Hummel ('59). Results are expressed as percentage inhibition of tryptic activity. The validity of this assay was determined using the trypsin inhibitor Aprotinin. This inhibitor was chosen as a standard, since its small molecular weight of 6,511 (Keesey, '87) would result in an elution profile similar to that expected for lamprey pancreatic trypsin inhibitor.

Similarly, chymotrypsin inhibitory activity was determined by incubating 100 μl aliquots of the chromatographic fractions with 100 μl of purified bovine chymotrypsin (50 μg ml^{-1}) for 10 min at 20°C and then assaying 100 μl of the mixture for chymotrypsin actively using the method described by Hummel ('59).

All assays were performed on at least three batches of diverticular extracts obtained in each case from between 10 and 15 ammocoetes, except for the assays for tryptic activity in *M. mordax,* when three batches of diverticular extracts from circa 50 animals in each case were used (see Results for rationale).

RESULTS

After rat pancreatic juice had been subjected to molecular sieve chromatography and the resultant fractions activated by incubation with exogenous enteropeptidase, trypsinogen eluted in a narrow band, with a peak at fraction 26 (Fig. 1A). The activity of the trypsin inhibitor likewise eluted in a narrow band, but with a peak at fraction 47 (Fig. 1B).

After subjecting the chromatographic fractions of diverticular extracts of *Geotria australis* to the same activation procedure as above, trypsinogen eluted with a sharp peak in the same position as that of rat pancreatic juice, i.e., fraction 26 (Fig. 2A). The activity of this peak corresponded to 13.3 μmoles min^{-1} g^{-1} tissue. There was no evidence of tryptic inhibitory activity, even at fraction 47 where the trypsin inhibitor of rat pancreatic juice eluted (cf. Figs. 1B, 2B).

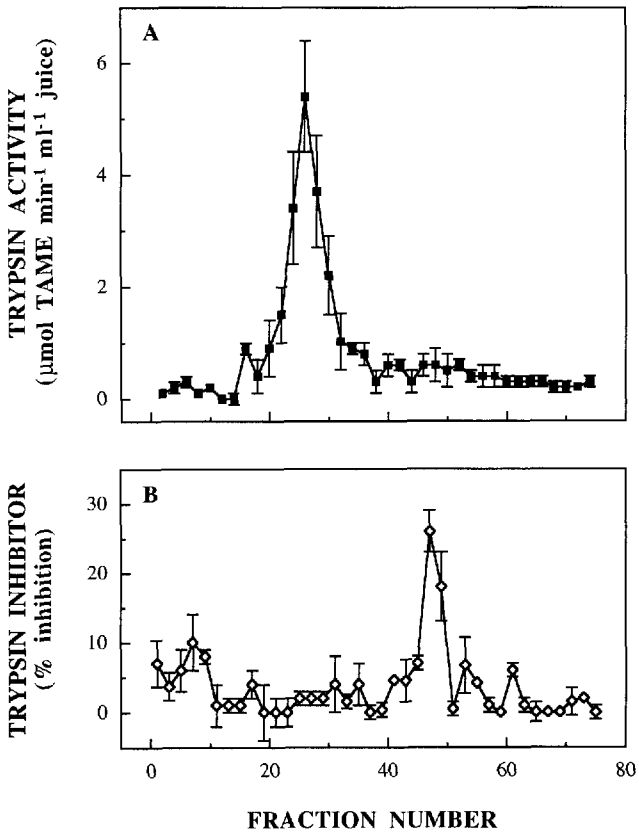

Fig. 1. Mean values ± SEM for **(A)** trypsin activity and **(B)** percentage inhibition of trypsin by a trypsin inhibitor in chromatographic fractions derived from rat pancreatic juice.

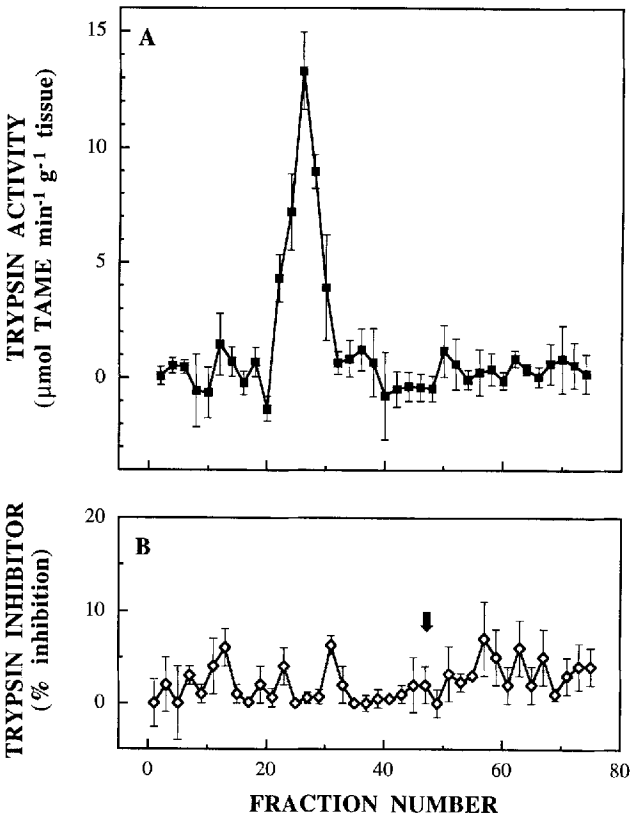

Fig. 2. Mean values ± SEM for **(A)** trypsin activity in chromatographic fractions of diverticular extracts from 10–15 larval *Geotria australis*. **B** shows that there is no evidence of a trypsin inhibitor in the same extracts. The arrow denotes the position where rat trypsin inhibitor elutes.

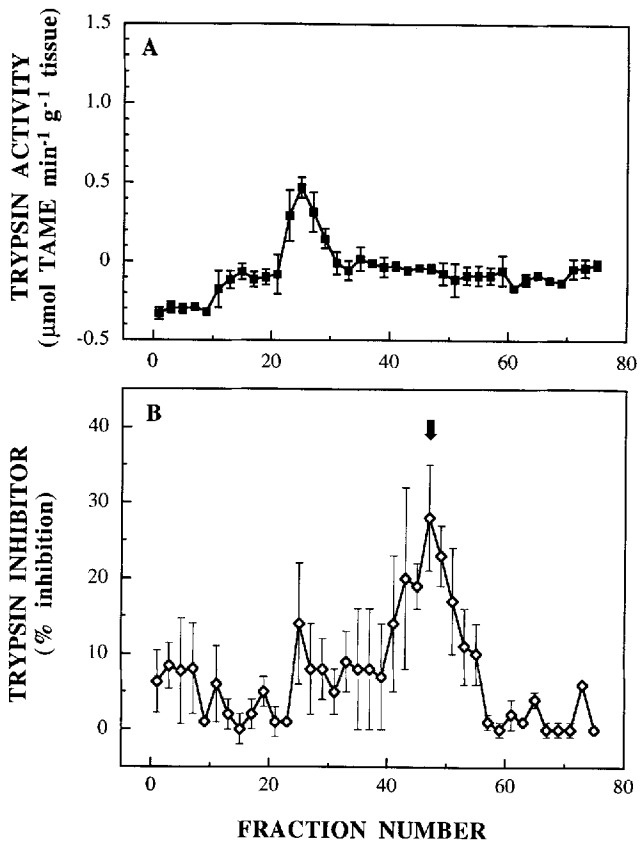

Fig. 3. Mean values ± SEM for **(A)** trypsin activity and **(B)** percentage inhibition of trypsin by trypsin inhibitor in chromatographic fractions derived from diverticular extracts of larval *Mordacia mordax*. The arrow shows that the trypsin inhibitor of *M. mordax* elutes in the same position as that of the rat. N.B.: Extracts from approximately 50 and 10–15 diverticula were used for A and B, respectively.

When exposed to the same procedures as those used for *G. australis* diverticular extracts, and when employing material from the same number of animals, i.e., 10–15, diverticular extracts of *Mordacia mordax* showed no detectable trypsinogen activity (data not shown). However, tryptic inhibitory activity produced a conspicuous peak at the same fraction (47) as that of rat pancreatic juice (cf. Figs. 1B, 3B). The range of fractions over which the inhibitor eluted, however, was greater in *M. mordax* than in the rat.

An inability to detect trypsinogen activity after exposing diverticular extracts of *M. mordax* to exogenous enteropeptidase was surprising in view of the fact that (1) an inhibitor of trypsin was present and (2) trypsin is the only known activator of chymotrypsinogen, which has been shown to be present in the diverticulum of this species (Cake et al., '92). Since a far greater amount of tissue may be required to reveal trypsinogen in diverticular extracts of *M. mordax* than in those of *G. australis,* the amount of tissue was increased by raising from 10–15 to circa 50 the number of animals from which diverticula were extracted for each individual analysis. Chromatographic analysis of the diverticular extract obtained using this far greater mass of tissue revealed a small but well defined peak of trypsinogen activity at fraction 25 (Fig. 3A). The activity of this peak corresponded to 0.5 μmoles min^{-1} g^{-1} tissue.

The diverticular extracts of *M. mordax* showed no chymotryptic inhibitory activity in those fractions of the chromatogram where the trypsin inhibitor elutes (Fig. 4B). However, there was a dip in the profile at fraction 21, corresponding closely to the position where chymotrypsinogen elutes (Fig. 4A). This clearly indicates that some of the chymotrypsinogen had been activated during incubation, presumably as a result of the tryptic activity that is present in these fractions.

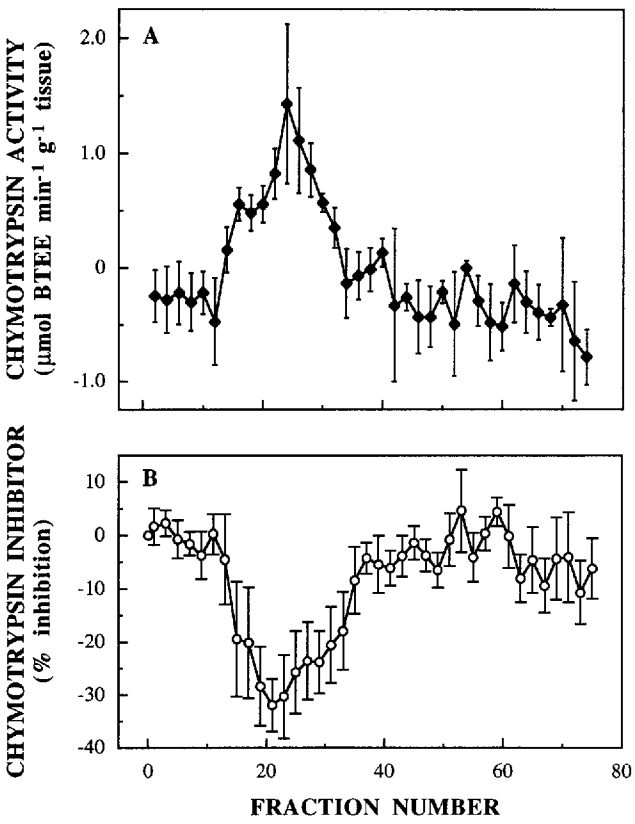

Fig. 4. Mean values ± SEM for **(A)** chymotrypsin activity in chromatographic fractions of diverticular extracts from 50 larval *Mordacia mordax*. **B** shows that there is no evidence of a chymotrypsin inhibitor in the same extracts. The large negative values around fraction 21 reflect prior activation of chymotrypsinogen in these fractions.

DISCUSSION

The results of this study confirm the hypothesis that a trypsin inhibitor is present in the intestinal diverticulum of larval *Mordacia mordax* and that it is not found in the two intestinal diverticula of larval *Geotria australis*. The lack of a trypsin inhibitor in the exocrine pancreas of the latter species is probably related to the consequences of the particular structure of its diverticula. It is proposed that the wide unfolded, tube-like diverticula of *G. australis* would facilitate the relatively rapid dispersal of trypsin into the anterior intestine and, as a consequence, the cells lining the diverticula would be exposed to trypsin for only short periods and would thus not require protection by a trypsin inhibitor. Since the ammocoetes of northern hemisphere species do not possess intestinal diverticula and the exocrine zymogen cells are thus located in the epithelial lining of the anterior intestine, it seems highly probable that they also do not possess a trypsin inhibitor. Such a conclusion is strongly supported by the fact that, in contrast to the situation with *Mordacia*, the chymotrypsinogen in anterior intestinal extracts of holarctic ammocoetes is already fully activated prior to exposure to exogenous trypsin (Cake et al., '92). Furthermore, since the zymogen cells in ammocoetes of holarctic species discharge their products directly into the intestine, there would be no requirement for a secretory trypsin inhibitor of the type found in *Mordacia*.

In contrast to the situation in the diverticula of *G. australis*, the highly folded internal epithelial lining of the diverticulum of larval *M. mordax* would presumably tend to slow down the release of the trypsin from the narrow convoluted lumina of the diverticulum into the intestine. A trypsin inhibitor would thus help protect the epithelial lining from its own proteolytic secretions. It is also noteworthy that, in comparison with the relatively high activities recorded previously for other digestive enzymes in the diverticulum of *M. mordax*, i.e., chymotrypsin, amylase, and lipase (Cake et al., '92), the activity of trypsin determined in this paper in *M. mordax* is relatively very low. Indeed, the activity is less than 4% of that recorded in the diverticula of *G. australis*. This low tryptic activity suggests that trypsin plays at best a minor role in protein digestion, but it still must be sufficiently high to induce the activation of chymotrypsinogen and other zymogens. Our results indicate that the potentially deleterious effects of exposing the epithelial cells of the diverticulum of *M. mordax* to their own proteolytic secretions are alleviated by the presence not only of a trypsin inhibitor but also by a low level of tryptic activity.

Our study shows that the inhibitor of the diverticulum of *M. mordax* inhibits trypsin but not chymotrypsin. It is thus a Kazal-type rather than a Kunitz-type inhibitor (Pubols et al., '74; Rinderknecht, '86). Thus, the trypsin inhibitor of *M. mordax* is presumably analogous to the pancreatic secretory trypsin inhibitor (PSTI) of other vertebrates. In mammals, this inhibitor, together with the zymogens, is secreted by the exocrine acinar cells into the pancreatic juice, where it forms an inactive complex with trypsin, thus preventing the premature trypsin-catalyzed activation of the other zymogens within the pancreas and pancreatic duct.

It has been suggested that each of the two southern hemisphere families of lampreys have evolved separately from stocks of holarctic lam-

preys (Petromyzontidae), similar to those represented in the contemporary fauna by *Ichthyomyzon unicuspis* (Hubbs and Potter, '71; Potter and Hilliard, '87). Since there is also clear evidence that the Mordaciidae is the most divergent and in some ways the most specialized of these families, it may well have diverged earlier than the Geotriidae (Potter, '86; Potter and Hilliard, '87). The location of exocrine cells in the lining of the anterior intestine of larval lampreys, as is the case in petromyzontids, has long been considered the most primitive arrangement of these zymogen cells found within the vertebrates (Brachet, 1897; Barrington, '72). It would then follow that, during the evolution of the Geotriidae, the zymogen cells became largely confined to left and right diverticula, which were developed as prominent tubular evaginations of the proximal part of the anterior intestine (Hilliard and Potter, '88; Bartels and Potter, '95). It seems likely that the single diverticulum of *M. mordax* evolved initially as a similar tube-like extension, with the internal epithelial lining later undergoing folding and containing essentially all of the zymogen cells (Hilliard and Potter, '88; Bartels and Potter, '95). A trypsin inhibitor and a reduced level of trypsin activity are thus assumed to have evolved as protective mechanisms against proteolysis in these folds.

The above proposed trends for the evolution of the diverticular, exocrine pancreas amongst the living lampreys contrasts with that proposed by Epple and Brinn ('87), who consider that the diverticula underwent regression during evolution, thereby accounting for why the exocrine pancreatic cells are found in the anterior intestine in larval petromyzontids. However, our scheme is entirely consistent with views regarding the interrelationships between the three extant lamprey families. It implies that, during the evolution of the protopancreas of *Mordacia* from a less specialized arrangement of exocrine pancreatic cells, a trypsin inhibitor was developed independently of the similar-sized pancreatic trypsin inhibitor of gnathostomes. This should not be considered too unlikely since the Kazal and Kunitz trypsin inhibitors found in the exocrine pancreas of bovids are of essentially the same molecular size (Burck, '70; Huber et al., '71; Keesey, '87). Furthermore, the complex structure of the exocrine pancreas of *Mordacia* differs from those typically found in gnathostomatous fish (Vonk and Weston, '84). Moreover, if a complex exocrine pancreas, containing an inhibitor, had been present in the common ancestor of both lampreys and gnathostomes, it would then have had to have been lost entirely in northern hemisphere lampreys and undergone internal changes in *Geotria*, with this latter genus then also developing a second smaller and left diverticulum. Apart from the fact that these latter trends would run entirely counter to contemporary views regarding the evolution of the different genera within the lamprey group (Hubbs and Potter, '71; Potter and Hilliard, '87), it is hard to envisage the type of selection pressures that would have resulted in a complex exocrine pancreas of the *Mordacia* type undergoing the modification required to produce the different arrangements of the zymogen cells now exhibited by the ammocoetes of holarctic lampreys and *Geotria*.

The question now arises as to the adaptive significance of the development of exocrine diverticular extensions by the ammocoetes of southern hemisphere lampreys. Such diverticula would focus the digestive enzymes at the proximal end of the anterior intestine, rather than along that part of the alimentary canal. The development of prominent diverticula, especially when the internal walls become highly folded as in *Mordacia*, would also be likely to increase the amount of digestive enzymes produced. Since ammocoetes frequently ingest large amounts of detritus (Moore and Mallatt, '80; Sutton and Bowen, '94), the possession of exocrine diverticula could be of particular value to the larvae of southern hemisphere lampreys, as they live in an environment in which the leaf material entering the streams and rivers, from for example *Eucalyptus* and *Notofagus,* is inherently tough and slow to decompose (Bunn, '86; Winterbourne, '95; Winterbourne, personal communication).

In summary, earlier workers (e.g., Brachet, 1897; Barrington, '72) considered that the location of zymogen cells in the epithelial lining of the anterior intestine of holarctic ammocoetes represents the most primitive arrangement of these exocrine pancreatic cells in vertebrates. We also consider that this arrangement closely resembles that found in ammocoetes of early lampreys. It thus follows that the intestinal diverticula of *Mordacia* and *Geotria*, which contain all or most of the zymogen cells, represent forward-projecting evaginations of the proximal part of the anterior intestine of such an ancestral ammocoete. These diverticula are presumed to increase the efficiency of digestion. The fact that a trypsin inhibitor is present in the diverticulum of *Mordacia*,

but not in those of *Geotria*, implies that it is required to help protect from proteolysis the highly folded epithelial lining that characterizes this diverticulum. If the above line of argument is accepted, it then also follows that a trypsin inhibitor has been evolved within the protopancreas of *Mordacia*, independently of that of the pancreas of gnathostomes.

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