8. SUMMARY

The exocrine pancreatic ribonucleases form a group of relatively rapidly evolving proteins. Their distribution among the vertebrate species is exceptionally unequal; an acceptable explanation of this distribution by a possible physiological function has been suggested only for animals with ruminant or ruminant-like digestive systems. Many ribonucleases are glycosidated to a greater or lesser extent.

Bovine ribonuclease is one of the most thoroughly studied enzymes. Comparative studies with ribonucleases derived from other species may yield valuable information on their phylogeny and structure-function relationships, and on the specificity requirements for the types of oligosaccharide side-chain attached.

Crude horse ribonuclease was isolated from fresh tissue by acid extraction, phenol extraction and ammonium sulfate fractionation (4.1). Further purification of the enzyme was found to be difficult. After trying many modifications, we succeeded by using repeated chromatography on CM-cellulose with three different gradient systems (4.2). The purity of the preparations was checked by disc gel electrophoresis and "staining" for ribonucleolytic activity, and by N-terminal analysis (4.3, 4.5); the specific activity was found to be an unreliable criterion for purity.

Pure horse ribonuclease was both chromatographically and electrophoretically heterogeneous. We have made plausible that the heterogeneity resides in the carbohydrate moiety (4.8).

The amino acid composition of horse ribonuclease has the characteristics common to the other known pancreatic ribonucleases. Tryptophan is absent. The carbohydrate moiety contains glucosamine and hexoses (4.4). Determination of the molecular weight was difficult because of the carbohydrate moiety; by a combination of methods we found a value of 14,000 for the molecular weight of the protein moiety and about 18,000 for the average molecular weight of the glycoprotein (4.7). Horse ribonuclease has the usual N-terminus lysine, but its C-terminus is threonine instead of the usual valine. With spectrophotometric titration we found only two abnormal tyrosines and three normal ones (4.6).

We derived four series of peptides from horse ribonuclease (Chapter 5): TC (tryptic and chymotryptic digestion after performic acid oxidation), T (tryptic cleavage after performic acid oxidation), CN (cyanogen bromide cleavage followed by performic acid oxidation), and AET (tryptic hydrolysis after reduction and aminoethylation). Some secondary cleavages were performed with thermolysin, chymotrypsin, and cyanogen bromide. Peptides were isolated and purified by gel filtration, chromatography on various ion exchangers, and column electrophoresis. The latter method proved particularly useful for glycopeptides (3.5.5). For the detection of peptides in column eluates we used mainly spectrophotometric monitoring, manual ninhydrin analysis, and automated analysis with a considerably modified peptide analyser that has been described at some length (3.5.7). The purity of the peptides was checked by high voltage paper electrophoresis, amino acid analysis and dansylation. Peptide sequences were determined by conventional methods: two versions of the Dansyl-Edman technique and a modification of the direct Edman degradation (3.6).

Only the AET-peptides covered the entire sequence. Several of the CN-peptides had arisen from aspecific cleavages, especially to the right of tyrosine residues. All peptides were positioned by sequence homology and overlappings; all but four peptide bonds were overlapped by one or more peptides (6.2). Almost all amide groups were located unequivocally (6.1).

Horse ribonuclease consists of a polypeptide chain containing 125 amino acid re idues and carrying three oligosaccharide side-chains in positions 21, 34, and 62. Part of Asn-21 occurs in the carbohydrate-free form (6.4). All three sites conform to the postulated recognition triplet for glycosidation; site 21-23 is the first attachment site in any glycoprotein found to contain proline (7.3).

The C-terminus of horse ribonuclease contains two additional amino acids, representing the only instance of a C-terminal addition in pancreatic ribonucleases observed thus far. Another peculiarity unprecedented in pancreatic ribonucleases is the deletion of residue 39. Including deletion and addition, horse ribonuclease differs in 35 positions from the bovine enzyme (7.1). All substitutions, the addition, and the oligosaccharide residues can be accommodated in the three-dimensional model of bovine ribonuclease-S without alterating the folding of the backbone. The model built of the loop containing the deletion offered an elegant explanation for the deviating titration behaviour of horse ribonuclease: the shortening and consequent distortion of this loop prevents Tyr-92 from forming a hydrogen bridge between its phenolic hydroxyl group and the carbonyl oxygen of residue 37 (7.2).

Comparison of all ribonuclease sequences elucidated partly or completely thus far provided the information to construct a preliminary evolutionary tree and a hypothetical ancestral amino acid sequence of mammalian pancreatic ribonuclease (7.1).

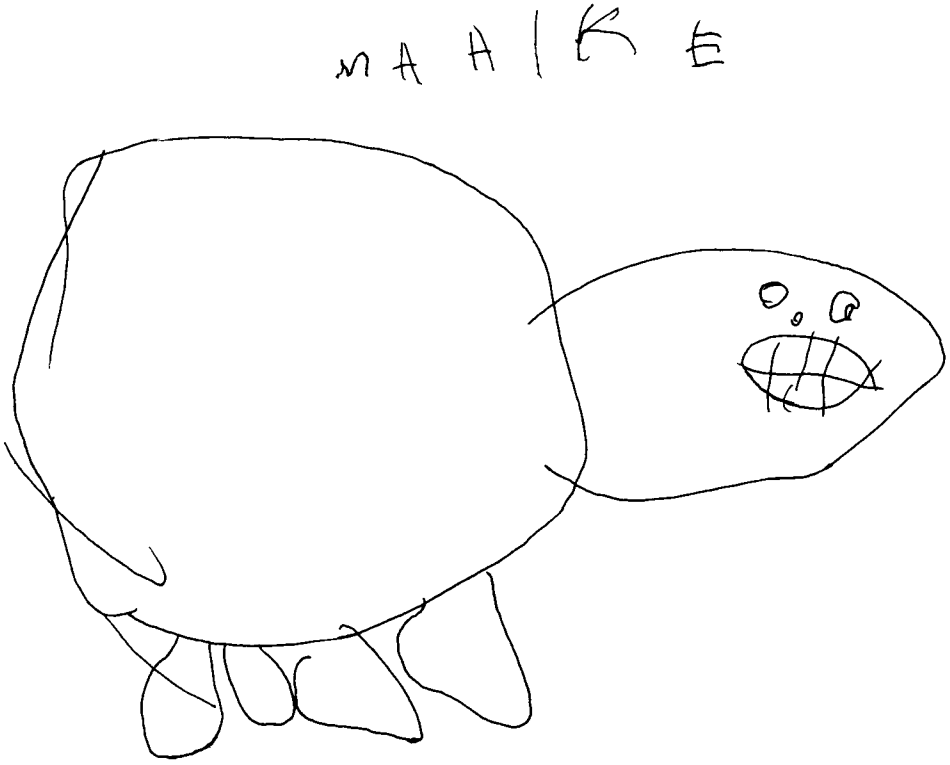

Fig. B. Ancestral mammal