Pancreatic Enzymes of the African Lungfish *Protopterus aethiopicus**

Gerald R. Reeck,† William P. Winter,‡ and Hans Neurath§

ABSTRACT: The major pancreatic enzymes or the respective zymogens of the African lungfish, *Protopterus aethiopicus*, have been chromatographically separated and identified. All pancreatic hydrolases known to occur in other vertebrates have been found. All proteolytic enzymes are present as zymogens, including three trypsinogens, two procarboxypeptidases A, and procarboxypeptidase B. In addition, three, possibly four, chymotrypsinogens are present, two of which cochromatograph with and may be identical with proelastase components. One other proelastase component, which does not exhibit chymotryptic activity after activation, is also observed. Two amylases, two ribonucleases, deoxyribonuclease, and trypsin inhibitor were also identified. Lipase activity was present when no diisopropyl phosphorofluoridate was added to the extraction slurry. Of particular interest are the rather strongly anionic character of the trypsinogens at pH 8.0 and the apparently monomeric state of the procarboxypeptidase A. Procarboxypeptidase B, however, cochromatographs with a chymotrypsinogen and is not fully separated from a trypsinogen so that a zymogen aggregate between procarboxypeptidase B and chymotrypsinogen and/or trypsinogen cannot be excluded.

The investigation of proteins of phylogenetically diverse species has greatly increased our knowledge of changes in enzyme structure and function in the course of evolution. The presence of a variety of hydrolytic enzymes in relatively high concentrations has made the exocrine pancreas an organ of choice for comparative studies of that kind. It is now well established that all major enzymatic activities in the pancreas can be separated from each other by chromatographic procedures (Keller *et al.*, 1958; Green *et al.*, 1963; Marchis-Mouren, 1965), but until recently the application of these techniques has been restricted almost entirely to mammalian species. The only extensive investigation of lower vertebrates has been the study of the pancreatic enzymes of the spiny Pacific dogfish (Prahl and Neurath, 1966; Lacko and Neurath, 1967). In continuation of our interests in the alteration of protein structure in the course of evolution (Neurath *et al.*, 1967, 1969) we report here on the pancreatic enzymes of the African lungfish, *Protopterus aethiopicus*.

The lungfish are a group of air-breathing, fresh-water fish found in swampy waters on three continents, Africa, South America, and Australia. *Protopterus* is one of the three major genera, all of which are equipped with both functional gills and lungs. In relation to their complement of pancreatic enzymes, it is of interest to note that all lungfish are carnivores.

The lungfish, of the order of Dipnoi, have developed from the main branch of vertebrate evolution which subsequently gave rise to amphibia and higher vertebrates. The lungfish thus represent a later stage of development than the dogfish, and like the latter, are prime examples of evolutionary constancy, having changed little morphologically from their ancestral forms.

The present report describes the chromatographic separation and enzymatic identification of the major pancreatic enzymes, or their respective zymogens, of the African lungfish.

Experimental Procedure

Materials. TISSUE. The collection and preparation of the lungfish tissue used in this work was carried out by Tiburon Biomarine Preparations, Edmonds, Wash., under the direction of Mrs. Susan Brown. The internal viscera from fish netted in Uganda, Africa, were removed and shipped frozen. Upon arrival, these were thawed and the pancreas dissected free of extraneous tissue. Acetone powder preparations of the pooled glands were made using the procedure of Wintersberger *et al.* (1962) except that ether was omitted from the final step. The powder was air dried until no acetone odor remained, and stored at −20° until used.

SUBSTRATES AND REAGENTS. *p*-Tosyl-L-arginine methyl ester was purchased from Cyclo Chemical Corp. Benzoyl-L-tyrosine ethyl ester was a product of Calbiochem. Carbobenzoxyglycyl-L-phenylalanine was obtained from Eli Lilly and Co. Orcein-impregnated elastin and salmon sperm DNA were products of Worthington Biochemical Corp. as was once-crystallized bovine trypsin, containing 50% $MgSO_4$. [^{3}H]TMV RNA was a gift from Dr. Milton P. Gordon. β-Naphthyl nonanoate was a product of Nutritional Biochemicals Corp. DFP was purchased from Merck Chemical Co.

Methods. ASSAY PROCEDURES.[1] Tosyl-L-arginine methyl ester

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[1] These enzymatic assays and the substrate specificities of well-characterized pancreatic enzymes from other species form the basis for the tentative designation of zymogens in this paper.

was employed to assay spectrophotometrically for trypsin activity (Hummel, 1959). Benzoyl-L-tyrosine ethyl ester was used to evaluate chymotrypsin activity in a spectrophotometric assay similar to that of Hummel (1959) except that the substrate was dissolved directly in a buffer containing 0.05 M $CaCl_2$ and 0.04 M Tris, pH 7.8, by heating and shaking vigorously, and no ethanol was used.

Exopeptidase activity was determined using carbobenzoxyglycyl-L-phenylalanine and benzoylglycyl-L-arginine as substrates for carboxypeptidases A and B, respectively. The hydrolysis of carbobenzoxyglycyl-L-phenylalanine was followed by measuring the decrease in absorbance at 225 nm of a 1×10^{-3} M solution containing 0.01 M NaCl and 0.005 M Tris, pH 7.5 (Whitaker *et al.*, 1966). Activity toward benzoylglycyl-L-arginine was measured in a spectrophotometric assay according to Folk *et al.* (1960) except that a substrate concentration of 5×10^{-4} M was used. In the above assays, a unit of enzymatic activity is that amount of enzyme which hydrolyzes 1 μmole of substrate in 1 min under the conditions of the assay.

Orcein-impregnated elastin was used to measure elastase activity as described by Sachar *et al.* (1955) except that a 1-hr incubation was used. A unit of activity is that amount of enzyme which solubilizes 1 mg of elastin in 1 hr. Ribonuclease activity was determined as follows: a 0.1-ml sample of the solution to be assayed was added to a mixture containing 20 μl of 1 M potassium phosphate, pH 7.0, 25 μl of a solution of bovine serum albumin (10 mg/ml), 20 μl of 0.05 M $MgSO_4$, 30 μl of H_2O, and 5 μl of [^{3}H]TMV RNA with total counts per minute of 4440. This was incubated for 30 min at 37°, and the reaction was then stopped by addition of 0.2 ml of 10% trichloroacetic acid. After centrifuging for 10 min in a desk top centrifuge, 0.2 ml of the supernatant was withdrawn and counted in a Packard-Tricarb scintillation counter. Results are expressed as percent of total potential radioactivity released.

Deoxyribonuclease activity was determined as described by Kunitz (1950). A unit of activity is that amount of enzyme which causes an increase in absorbance of 0.001/min per ml of reaction mixture. An assay described by Kramer *et al.* (1963) and modified by Bradshaw (1968) using β-naphthyl nonanoate as substrate was used to evaluate lipase activity. Amylase activity was determined according to the method of Bernfeld (1951). A unit of activity is that amount of enzyme which liberates 1 μmole of reducing groups calculated as maltose per minute.

Trypsin inhibitor was assayed by incubating 100 μl of the sample to be assayed with 5 μg of bovine trypsin and 10 μl of 1 M Tris at pH 8.0 for 1 hr at room temperature. The incubation mixture was then assayed for activity toward tosyl-L-arginine methyl ester and the results are expressed as percent of inhibition of the bovine trypsin.

All spectrophotometric assays were performed with a Gilford Model 2000 spectrophotometer.

Fractions to be assayed for activity toward tosyl-L-arginine methyl ester, benzoyl-L-tyrosine ethyl ester, carbobenzoxyglycyl-L-phenylalanine, or benzoylglycyl-L-arginine were routinely treated with bovine trypsin by adding 40 μg of trypsin, 50 μl of 1 M $CaCl_2$, and 50 μl of 1 M Tris, pH 8.0, to 0.5 ml of sample. In regions where trypsin inhibitor was detected 200 μg of bovine trypsin was utilized to ensure complete activation of all zymogens. Activities were followed until a maximum was reached. This required less than 0.5 hr in every case except that of the trypsin activity, which required over 2 hr.

In the assay for elastase activity, 200 μg of bovine trypsin was added at the start of the incubation to generate potential enzymatic activity.

Column Chromatography and Gel Filtration Media. DE-52 and CM-32 ion-exchange celluloses were prepared and packed as advised by the manufacturer (Whatman). Sephadex G-100, purchased from Pharmacia, was used as recommended by the manufacturer for gel filtration.

Extraction. Acetone powder was extracted by stirring 10 ml of water/g of powder, for 4 hr at 4°. One drop of octanol was included to suppress foaming. The slurry was brought to 1×10^{-3} M DFP initially and again after 2 hr of extraction. After extraction, the slurry was centrifuged for 15 min at 30,000*g* to remove insoluble material. The pH of the supernatant was approximately 6.5.

Results

Preliminary Observations. The pancreas of *P. aethiopicus* is black while fresh, in contrast to the pinkish appearance of other vertebrate pancreata, and yielded a slate-gray acetone powder. Extraction and centrifugation of the slurry gave a brown supernatant and a pellet of black residue covered with a thin white layer.

During the dialysis of the sample immediately preceding the initial chromatography, an unidentified yellow pigment diffused into the buffer; a small amount of yellow pigment also appeared in the breakthrough in the initial chromatography. A reddish brown pigment adhered to the anion-exchange cellulose in the initial chromatography and was not removed by any treatment tried.

When extraction was carried out as described above, no trypsin, chymotrypsin, or carboxypeptidase activity was observed prior to activation. When no DFP was added during the extraction, however, nearly 30% of the total activatable carboxypeptidase was detected as were low levels of trypsin and chymotrypsin. Trypsin inhibitor, ribonuclease, and amylase activities were detected prior to activation, as was lipase when no DFP was added during the extraction. Portions of extracts were then prepared and chromatographed successively on anion and cation exchangers.

Anionic Proteins. The aqueous extract of 3 g of acetone powder prepared as described in the Methods section was brought to 70% saturation by the addition of solid ammonium sulfate at 0°, pH 6.5. The sample was then centrifuged for 15 min at 30,000*g*, the pellet of precipitated protein dissolved in 20 ml of water, and 10 μl of 5 M DFP added. At this stage, the A_{280}:A_{260} ratio was greater than 1.60, indicating no appreciable contamination with nucleic acid. The solution was then dialyzed overnight against 16 l. of 0.005 M NaCl, 0.005 M potassium phosphate, pH 8.0, the starting buffer for the initial chromatography. After dialysis, the sample was once again treated with 10 μl of 5 M DFP and allowed to stand for about 2 hr before application to a 2.5 × 40 cm column of DE-52. The column was developed in the cold room at a flow rate of 100 ml/hr using a Technicon pump. After elution of 1200 ml, a 2000-ml linear gradient of 0.005–0.20 M NaCl in 0.005 M potassium phosphate buffer, pH 8.0, was applied followed by 500 ml of 2.0 M NaCl in the same phosphate buffer.

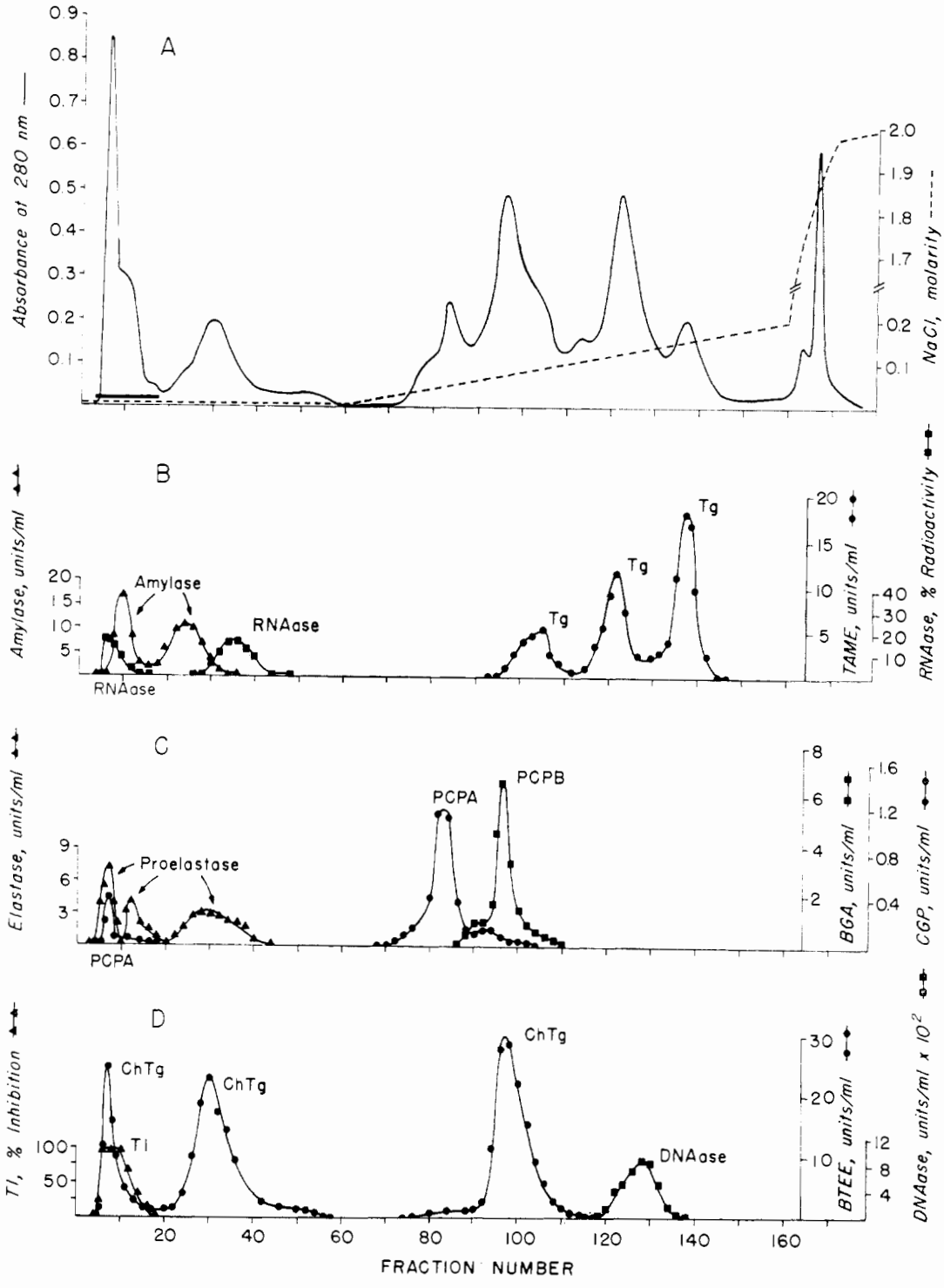

FIGURE 1: Chromatography on DE-52 (DEAE-cellulose) of an aqueous extract of acetone powder of lungfish pancreas. Details of the chromatography are given in the text; fraction volume 20 ml. Enzymatic assays were performed both before and after treatment with bovine trypsin. Results are shown only in those regions in which activity was detected. Ribonuclease, deoxyribonuclease, and amylase activities were independent of treatment with trypsin; all other enzymatic activities were present only after such treatment. Breakthrough fractions indicated by a bar were pooled for subsequent chromatography on CM-cellulose (Figure 2). Abbreviations used are: RNAase, ribonuclease; Tg, trypsinogen; PCPA, procarboxypeptidase A; PCPB, procarboxypeptidase B; TI, trypsin inhibitor; DNAase, deoxyribonuclease; BGA, benzoylglycyl-L-arginine; BTEE, benzoyl-L-tyrosine ethyl ester; CGP, carbobenzoxyglycyl-L-phenylalanine; TAME, tosyl-L-arginine methyl ester. (A) Elution profile and NaCl gradient; (B) assays for amylase, ribonuclease, and trypsin; (C) assays for carboxypeptidases A and B and elastase; (D) assays for trypsin inhibitor, chymotrypsin, and deoxyribonuclease. Assays are grouped as shown solely for clarity of presentation.

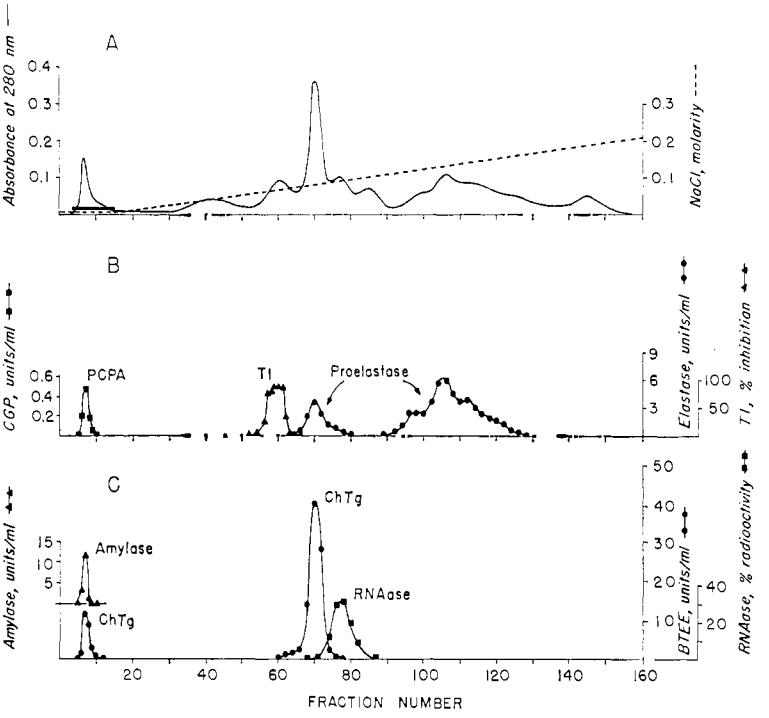

FIGURE 2: Chromatography on CM-32 (CM-cellulose) of the breakthrough fractions of the DE-52 chromatography of Figure 1. Details of the chromatography are given in the text; fraction volume 10 ml. Enzymatic assays were performed both before and after treatment with bovine trypsin. Results are shown in only those regions in which activity was detected. Ribonuclease and amylase activities were independent of treatment with trypsin. All other enzymatic activities were present only after such treatment. Breakthrough fractions indicated by a bar were pooled for subsequent gel filtration (Figure 3). For abbreviations, see legend to Figure 1. (A) Elution profile and NaCl gradient; (B) assays for trypsin inhibitor and for carboxypeptidase A and elastase activities; (C) assays for amylase, chymotrypsin, and ribonuclease.

The elution profile of chromatography on DEAE-cellulose DE-52 is shown in Figure 1A in terms of A_{280}. This pattern was entirely reproducible and the recovery of material absorbing at 280 nm was consistently better than 90%. The column fractions were assayed before and after incubation with bovine trypsin. Activities corresponding to ribonuclease, deoxyribonuclease, and amylase were independent of treatment with trypsin. Figures 1B, C, and D display the enzymatic activities observed after activation. The peak emerging after application of 2.0 M NaCl (Figure 1A) was of variable height and devoid of enzymatic activity before and after treatment with bovine trypsin. Its strongly anionic character and low A_{280}:A_{260} ratio of 0.65 suggest that it contains nucleic acids.

Cationic Proteins. The breakthrough fractions resulting from chromatography on DE-52 (Figure 1) were pooled, lyophilized, dissolved in 0.005 M potassium phosphate, pH 6.0, containing 0.005 M NaCl, and dialyzed overnight against a large excess of the same buffer. The dialyzed sample was centrifuged to remove insoluble material and applied to a column of CM-cellulose CM-32 (1.5 × 100 cm). The column was developed in the cold room at a flow rate of 35 ml/hr and a linear gradient of NaCl from 0.005 to 0.3 M in total volume of 2.0 l. was applied. The elution profile and associated enzymatic assays are shown in Figure 2. The chromatography was entirely reproducible and recovery of material absorbing at 280 nm was greater than 90%. As in the previous chromatography, no activity toward substrates of proteolytic enzymes could be detected in any fraction prior to activation with trypsin. Procarboxypeptidase A, amylase, and chymotrypsinogen were detected in the breakthrough fractions. In addition, activities corresponding to chymotrypsinogen, proelastase, and ribonuclease were identified in the later fractions. Separation of amylase from the zymogens in the breakthrough fraction could be accomplished by gel filtration on a column of G-100 (2.5 × 110 cm) as shown in Figures 3A and B, whereas procarboxypeptidase A and chymotrypsinogen were incompletely separated from each other under these conditions. Rat amylase has been reported by Vandermeers and Christophe (1968) to elute similarly from Sephadex G-100.

Discussion

As a general observation, it seems significant that every pancreatic enzyme or zymogen that occurs in the bovine has been found in the lungfish. With the possible exception of lipase, only the proteolytic enzymes are present as zymogens, whereas all other hydrolases are fully active prior to incubation with trypsin. Activity toward the lipase substrate β-naphthyl nonanoate was observed only in preparations not treated with DFP. Such preparations give elution profiles very similar to those resulting from chromatography of samples treated with DFP, but the zymogens, especially procarboxypeptidase A, are partially activated. Under these conditions, lipase activity emerges between the second and third trypsinogen peaks (Figure 1C) and is independent of trypsin treatment. Lipase activity is apparently not affected by taurocholate, which has been reported to alter activity of mammalian lipases (Kramer *et al.*, 1963; Bradshaw, 1968). In the DFP-treated samples, no lipase activity is observed before or after treatment of the fractions with bovine trypsin, indicating that the enzyme probably is not present in the zymogen form.

It is worthy of note that certain enzyme activities are associated with more than one protein fraction. For instance, there appear to be two amylases, two ribonucleases, three—possibly four—chymotrypsinogens, three trypsinogens, and two procarboxypeptidases A. After activation, elastase activity was observed in three protein fractions, two of which also exhibited potential chymotryptic activity. Since the present experiments were carried out on pooled pancreas glands, it is not possible to decide whether this multiplicity

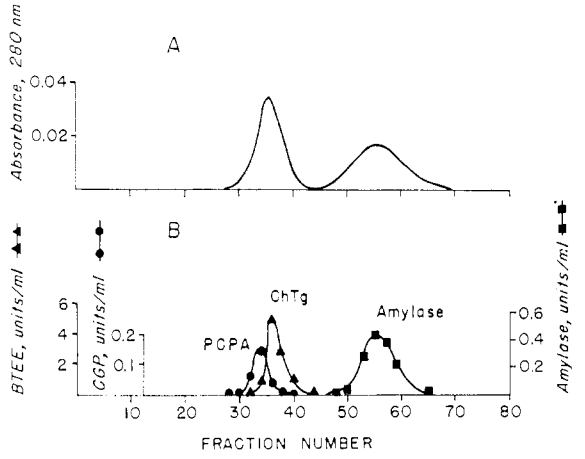

FIGURE 3: Gel filtration on Sephadex G-100 of the breakthrough fractions of chromatography on CM-32, shown in Figure 2. The pooled fractions were lyophilized and dissolved in 10 ml of the elution buffer, 0.005 M potassium phosphate, pH 6.0, containing 0.005 M NaCl, and this solution was brought to 5% sucrose by addition of solid sucrose. A flow rate of about 25 ml/hr was maintained by the hydrostatic pressure of a 40-cm head. The column size was 2.5 × 120 cm; fraction volume 10 ml. For abbreviations, see legend to Figure 1. (A) Elution profile; (B) enzymatic assays.

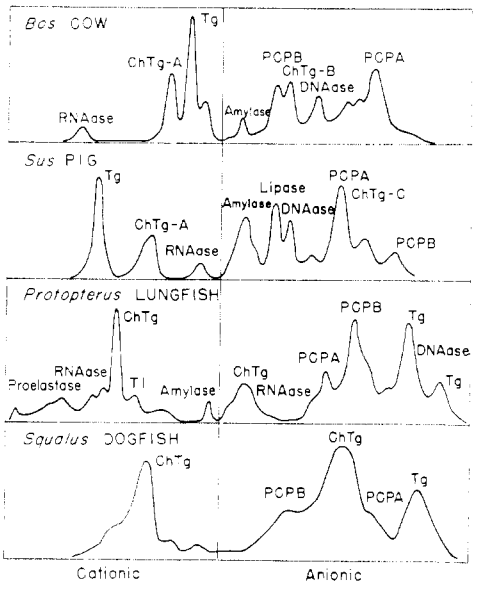

FIGURE 4: Composite elution profiles of pancreatic extracts of the cow, pig, lungfish, and dogfish. In each case the anionic proteins were chromatographed on an anion exchange column, and the breakthrough fractions (cationic proteins) from this column were chromatographed on a cation exchange column. The order of elution from the cation exchange column is reversed in each case so that the composite elution profiles show, from left to right, the most cationic to the most anionic proteins. For abbreviations, see legend to Figure 1. References to the individual chromatographic diagrams are given in Neurath *et al.* (1969).

is the result of allelomorphism, as in the case of bovine carboxypeptidase A (Walsh *et al.*, 1966; Pétra *et al.*, 1969), or due to the presence of multiple genes coding for different protein having the same biological function.

It has been shown that the chromatographic elution profile of pancreatic extracts from various species roughly parallels their phylogenetic proximity (Neurath *et al.*, 1969). For instance, as shown in Figure 4, the cow and the pig, both artiodactyls, are very similar to each other; both have pancreatic cationic ribonuclease, trypsinogen, and chymotrypsinogen, and both have anionic procarboxypeptidases A and B, anionic chymotrypsinogen, and anionic amylase and deoxyribonuclease. The order of elution differs, but the overall distribution is similar. The principle difference between the patterns of artiodactyls and of the lower vertebrates is the occurrence of strongly anionic trypsinogens. Both lungfish and dogfish exhibit at least one such acidic trypsinogen fraction, each possessing an anionic and a cationic chymotrypsinogen and anionic procarboxypeptidases, as do the artiodactyls. These variations appear to be phyletic characteristics, rather than expressions of biological function, which usually is considered to be a property of three-dimensional structure rather than a consequence of the surface charge of the molecule as such.

One of the characteristic properties of the bovine pancreas is the occurrence of aggregates between procarboxypeptidase A and a zymogen related to chymotrypsinogen (Yamasaki *et al.*, 1963). In the chromatogram of the lungfish, as of the dogfish, the major peak corresponding to procarboxypeptidase A is clearly separated from all chymotrypsinogens. The other peak associated with procarboxypeptidase A is asymmetrically disposed with respect to the adjacent chymotrypsinogen (Figure 3B). Preliminary high-speed sedimentation equilibrium experiments of this material fail to provide evidence for a species having a molecular weight greater than 45,000. It appears, therefore, that in the lungfish, as in the dogfish, procarboxypeptidase A occurs in monomeric form.

In contrast, procarboxypeptidase B cochromatographs with a chymotrypsinogen fraction and is not entirely separated from a trypsinogen fraction (Figures 1B, C, and D). Furthermore, in gel filtration on Sephadex G-100 at pH 9.0 and in ion-exchange chromatography on QAE-Sephadex at pH 9.0, procarboxypeptidase B elutes with chymotrypsinogen and trypsinogen. In addition, preliminary sedimentation velocity measurements on procarboxypeptidase B that had been further purified by either of the above techniques have yielded values of s_{20} between 5.6 and 6.0 at protein concentrations of approximately 10 mg/ml. These observations suggest that lungfish procarboxypeptidase B may occur as a zymogen aggregate with chymotrypsinogen and/or trypsinogen.

It is anticipated that the present chromatographic procedures will make possible the characterization of the various zymogens and enzymes of lungfish pancreas and to compare them to analogous proteins from other species. Such studies are now in progress.

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Studies of Lysosomal α-Glucosidase. I. Purification and Properties of the Rat Liver Enzyme*

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ABSTRACT: An α-glucosidase has been purified 1300-fold from the lysosomal fraction of rat liver. The preparation appears to be homogeneous when subjected to equilibrium ultracentrifugation. The degree of purification of the enzyme at each step in its preparation was essentially the same whether the assay substrate was maltose, glycogen, or isomaltose. Thus, the enzyme appears to have α-1,6-glucosidase as well as α-1,4-glucosidase activity. Both of these activities sedimented at the same rate when the purified enzyme was centrifuged in a linear sucrose gradient. The apparent molecular weight of the protein is about 114,000. The enzyme has maximal activity toward maltose at pH 3.7, toward isomaltose at pH 4.2, and toward glycogen at pH 4.4. Similar pH optima were found in acetate and citrate buffers. Glucose formation from glycogen can be stimulated fivefold at pH 4.0 by 0.2 M KCl. This stimulation was found for several monovalent and divalent cations. The influence of cation concentration on the rate of maltose and isomaltose hydrolysis is much less than on that of glycogen. The enzyme can act also as a transglucosylase at pH 4.0 when it is incubated with oligosaccharides such as maltose. Maltotriose and maltotetraose are formed together with a branched trisaccharide of unknown structure which has been shown to be distinct from panose and from 4,6-di-*O*-(α-D-glucopyranosyl)-D-glucose. The enzyme also catalyzes transglucosylation from maltose to glycogen. The structure of the polysaccharide product has been studied. Since the specific activity of the enzyme as a transglucosylase parallels its specific activity as a hydrolytic enzyme during purification, it is concluded that the lysosomal glucosidase has intrinsic transglucosylase activity.

The lysosomes which are present in liver are known to contain a large number of hydrolases with specificities directed toward a variety of intracellular and extracellular macromolecular substances as well as toward compounds of simpler structure which occur within the cell. Presumably, the metabolic role of these enzymes, whose activities are most pronounced under acidic conditions, is to convert their substrates into monomeric units so that these may again become available to the cell for biosynthetic and for energy-yielding reactions. This concept has been discussed in detail by de Duve and his coworkers (de Duve, 1965; de Duve and Wattiaux, 1966; Coffey and de Duve, 1968; Aronson and de Duve, 1968). The action on glycogen of enzymes within the lysosome is of special interest since Lejeune *et al.* (1963) showed that a lysosomal α-1,4-glucosidase, active at pH 4, is found in rat liver. Hers (1963) found that a similar activity is present in human liver, presumably within lysosomes as well. The further discovery was made by Hers (1963) that in children who have a fatal form of glycogen storage disease

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