Pancreatic Enzymes of the Spiny Pacific Dogfish. I. Cationic Chymotrypsinogen and Chymotrypsin*

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ABSTRACT: Investigation of the pancreatic enzymes of *Squalus acanthias*, a species phylogenetically distant from the bovine, has demonstrated that the pancreas of this species contains zymogens corresponding to the known mammalian proteolytic enzymes. These include cationic and anionic forms of chymotrypsinogens, an anionic form of trypsinogen, as well as zymogens of the carboxypeptidases A and B. By use of chromatographic procedures, the cationic chymotrypsinogen has been isolated, purified, and characterized by chemical and enzymatic procedures. The latter include determination of the molecular weight, isoelectric point, end groups, and amino acid composition. Activation by trypsin has been followed by end-group analysis and activity toward the synthetic substrate acetyl-L-tyrosine ethyl ester and the active enzyme, chymotrypsin, has been purified by chromatography. Dogfish chymotrypsin has been characterized by kinetic criteria, including pH and temperature dependence and by its inhibition by competitive inhibitors. Comparison with chymotrypsinogens derived from other species suggests that, in general, the "primeval" chymotrypsinogen resembles more closely the anionic form of the zymogen, and that the cationic forms in the fish and the higher mammals have evolved independently from this protein and its progeny.

Considerable experimental evidence has been obtained during the last few years to support the view that proteins which fulfill the same biological function have certain common molecular features. This was first demonstrated by Sanger and co-workers in their investigation of the amino acid sequence of insulin derived from various phyletic sources (Sanger, 1960; Randle, 1964). More recently, the phylogenetic relationship among hemoglobins in primates has been investigated by sequential amino acid analysis by Buettner-Janusch and Hill (1965), and an extensive study of the evolution of cytochrome *c* has been presented by Margoliash and Smith (1965). Detailed comparisons afforded by these investigations have enabled the delineation of those structural features which express biological function in contrast to those characteristic of the species. An alternative experimental approach to the relation between function and molecular structure of proteins is based on the comparison of certain catalytic and structural properties of enzymes of like specificity. Recent studies on aldolase (Rutter, 1965), on lactic dehydrogenase (Kaplan, 1965), and on phosphoglucomutase (Joshi *et al.*, 1965) are excellent examples of the success of the latter approach.

The proteolytic enzymes of the pancreas have a long history of investigation of the relation of chemical composition, structure, and biological function. Some of these, notably trypsin and chymotrypsin, were among the first enzymes wherein specific amino acid residues were implicated as being part of the active center (Balls and Jansen, 1952). With the recent completion of the amino acid sequence of two of these, bovine pancreatic chymotrypsinogen (Hartley, 1964) and trypsinogen (Walsh *et al.*, 1964), it has been possible to compare point by point the linear structure of these two enzyme precursors and to probe for analogies or homologies that might be related to function. This comparison has been recently extended to include certain peptide sequences in bovine chymotrypsinogen B and porcine trypsinogen and in porcine elastase (Rovery, 1964; Hartley *et al.*, 1965). To date, pancreatic proteolytic enzymes have been isolated from a variety of species besides the beef and the pig, *i.e.*, the dog, the horse, and the rat (Marchis-Mouren, 1959; Marchis-Mouren *et al.*, 1963). Proteolytic enzymes of the pancreas of avian origin have also been reported such as the chicken and the turkey (Ryan, 1965). The bovine and porcine systems in particular have served as models to examine in more detail similarities in structure that are related either to the mechanism of zymogen activation or to enzymatic function. Among the proteolytic enzymes that have been either isolated or else identified on the basis of substrate specificity are cationic as well as anionic chymotrypsin, cationic trypsin, carboxypepti-

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dases A and B, and elastase. An anionic form of trypsin to date has been reported only for the rat and more recently for the salmon (Croston, 1966). In view of the relatively close taxonomic relationship among mammals such as the beef, pig, or horse, it appeared of interest to examine the character of pancreatic proteolytic enzymes of a species whose evolutionary origin precedes the mammals by some 200,000,000 years. The salt water fish *Squalus acanthias* (commonly known as the Pacific spiny dogfish belonging to the order of *elasmobranchii*) appeared as an excellent object of study. This species, often referred to as a "living fossil," contains a distinct pancreatic gland whose fine structure is practically indistinguishable from that of the bovine (private communication by Dr. Edward Smuckler). This fish is indigenous to the Pacific Northwest and has been available for the present work in practically unlimited quantities. Preliminary studies have shown that aqueous extracts of an acetone powder of the pancreas of the Pacific spiny dogfish contains all the enzymatic activities, mostly in the zymogen form, found in bovine pancreatic extracts. The present paper describes the occurrence of the proteolytic enzymes in the dogfish pancreas and the isolation and characterization of cationic chymotrypsinogen and chymotrypsin. In a succeeding paper the isolation, characterization, and activation of dogfish procarboxypeptidase B will be presented (J. W. Prahl and H. Neurath, in preparation).

Experimental Section

Materials and Methods

Preparation of Pancreatic Acetone Powder. Dogfish pancreatic tissue was obtained from freshly collected living fish secured through commercial fishing trawlers in the Puget Sound area. After excision, the pancreas was immediately frozen in containers chilled with solid carbon dioxide. The frozen tissue was allowed to thaw overnight in a cold room at 4°. While still semisolid, the tissue was stripped of vessels, ducts, and connective tissue and washed with copious quantities of cold acetone. The tissue was then homogenized in a Waring blendor for approximately 30–45 sec. The homogenized material was suspended in cold acetone (approximately 200 g of fresh tissue/l. of solvent) and stirred for 4 hr. The suspension was filtered on a Büchner funnel and the precipitate was resuspended in an equal volume of cold acetone. This latter step was repeated twice. The powder was then suspended in an equal mixture of acetone and ethyl ether and finally in ethyl ether, both at room temperature. After the last filtration, the powder was dried in a desiccator and a gentle vacuum was applied with an aspirator. The powder was sealed in polyethylene bottles and stored at −20°. This procedure yielded approximately 1.6 g of acetone powder from 10 g of fresh pancreatic tissue. Powders thus prepared were stable for 18 months without undergoing autolysis or loss of activatability.

Substrates. Carbobenzoxyglycyl-L-phenylalanine[1] and benzoyl-L-arginine ethyl ester[1] were obtained from Mann Research Laboratories, Inc. Benzoyl-L-arginine *p*-nitroanilide[1] was a product of Nutritional Biochemicals Corp. Acetyl-L-tyrosine ethyl ester was purchased from Calbiochem. Benzoylglycine-L-arginine[1] was a gift from Dr. Roger Roeske of the Department of Biochemistry, University of Indiana Medical School. Hippuryl-D-L-β-phenyllactic acid was prepared by Dr. W. O. McClure (McClure and Neurath, 1966).

Reagents and Enzymes. Diisopropylphosphorofluoridate[1] was purchased from the Merck Chemical Co. and was diluted to 1 M with isopropyl alcohol before use. *Phenylmethanesulfonyl fluoride*[1] was purchased from Cyclo Chemical Corp. *Trypsin* containing 50% $MgSO_4$ was obtained as a twice-recrystallized product from Worthington Biochemical Corp. Before use, it was exhaustively dialyzed *vs.* 0.001 N HCl to remove salt. *Bovine-α-chymotrypsin*, three times recrystallized, was obtained from Worthington Biochemical Corp. *Bovine chymotrypsin B* was a gift of Dr. Michael Laskowski, Sr. *Bovine carboxypeptidase A* was prepared in this laboratory by the method of Cox *et al.* (1964). *Bovine carboxypeptidase B* was prepared by Dr. Erhard Wintersberger (Wintersberger *et al.*, 1962).

Succinyl trypsin was prepared according to the method of Fraenkel-Conrat (1949). Worthington bovine trypsin (300 mg), salt free, was dissolved in 8 ml of 50% saturated ammonium acetate. At 0°, 0.2 mg of succinic anhydride (Coleman) was slowly added to the stirring solution over a period of 1 hr. The reaction mixture was then dialyzed overnight *vs.* 0.005 M sodium succinate buffer, pH 5.0. The dialyzed protein was passed through a column of CM-cellulose which had been equilibrated with the same buffer, and the breakthrough fraction was collected and lyophilized.

Protein Concentration. The concentration of protein was routinely determined spectrophotometrically at 280 mμ. When the 280:260-mμ ratio was <1.65, correction for nucleic acid contamination was made according to Warburg and Christian (1941). Absorbancy indices ($A^{1\%}_{1cm,\ 280}$) of purified proteins were obtained by relating optical density to the concentration as determined in the ultracentrifuge using refractometric methods. A refractive index increment of 18.5×10^{-4} dl g^{-1} at 550 mμ was assumed (Doty and Edsall, 1951). These coefficients were checked where possible by amino acid analysis (Walsh and Brown, 1962).

Assay Procedures. Esterase activities were determined by null titration with 0.08–0.10 N standardized sodium hydroxide on the TTT-1 Radiometer autotitrator equipped with an Ole Dich recorder (Copenhagen) at 25°. ATEE was used to evaluate *chymotrypsin-like* activity (0.01 M ATEE, 0.1 M KCl, 0.01 M potassium

[1] The following abbreviations are used: CGP, carbobenzoxyglycyl-L-phenylalanine; BAEE, benzoyl-L-arginine ethyl ester; BAPA, benzoyl-L-arginine *p*-nitroanilide; ATEE, acetyl-L-tyrosine ethyl ester; BGA, benzoylglycyl-L-arginine; HPLA, hippuryl-DL-β-phenyllactic acid; PMSF, phenylmethanesulfonyl fluoride; PTC, phenylthiocarbamyl; FDNB, 1-fluoro-2,4-dinitrobenzene; STI, soybean trypsin inhibitor; ATryEE, acetyltryptophan ethyl ester; β-pp, β-phenylpropionate; DIP, diisopropylphoryl; TPCK, tosylphenylalanyl chloromethyl ketone.

phosphate buffer, pH 7.9). BAEE, 0.01 M (0.1 M KCl, 0.01 M potassium phosphate buffer, pH 7.9), was employed to assay for *trypsin-like* activity. Relative activity is expressed as the uptake of base in milliequivalents per minute per milliliter of enzyme solution or as base uptake in milliequivalents per minute per absorbance unit of 1.0 at 280 mμ. Specific activity is expressed as milliequivalents of base consumed per minute per milligram of protein.

A colorimetric assay using BAPA as substrate (Erlanger *et al.*, 1961) was employed to identify tryptic activity eluted during column chromatography. Activities are reported as absorbance units at 410 mμ per minute per milliliter of enzyme solution or absorbance units per minute per absorbance unit of 1.0 at 280 mμ.

Exopeptidase activity was determined using HPLA, CGP, or BGA as substrates. The hydrolysis of HPLA was determined according to Bargetzi *et al.* (1963). The release of phenylalanine from CGP was determined by development of the ninhydrin color (Putnam and Neurath, 1946). The hydrolysis of BGA was followed both spectrophotometrically (Folk *et al.*, 1960), and by the development of the ninhydrin color. The latter technique (0.003 M BGA in 0.005 M sodium Veronal and 0.045 M NaCl at pH 7.5) was employed to locate the activity in the eluent of chromatography. Peptidase activity is expressed as per cent hydrolysis per minute (units) per milliliter or as units per absorbance unit of 1.0 at 280 mμ; specific activity is expressed as units per milligram of protein.

Column Chromatography. Commercially available absorbents were employed for all column chromatographies (DEAE-cellulose, Brown, lot 1333, 0.93 mequiv/g; CM-cellulose, Brown, lot 1284, 0.80 mequiv/g; phospho(P)-cellulose, Serva, lot A1583, 0.77 mequiv/g; sulfoethyl(SE)-cellulose, Serva, lot A1629, 0.27 mequiv/g). After removing the fines, the absorbents were equilibrated, deaerated, and packed according to Peterson and Sober (1956). Linear and nonlinear gradients were applied (Peterson and Sober, 1960). DEAE-Sephadex A-50 (Medium, lot To 3024; Coarse, lot To 7874C) was prepared and packed as advised by the manufacturer (Pharmacia, Uppsala, Sweden).

Amino acid analysis was performed by the method of Spackman *et al.* (1958) on the Spinco amino acid analyzer Model 120. Acid hydrolysis was carried out in 5.7 N redistilled HCl *in vacuo* at 104° for periods of 24, 48, 72, and 98 hr. Norleucine was incorporated as an internal standard into each sample (Walsh and Brown, 1962). After hydrolysis, the samples were dried and taken up in 0.2 N sodium citrate buffer pH 2.2 containing a standard mixture of β-thienylalanine and L-2-amino-3-guanidopropionate. Cystine and cysteine were analyzed as cysteic acid after oxidation of an aliquot with performic acid according to Moore (1963), and the cysteic acid found was related to recoveries of alanine, glycine, and phenylalanine. Tryptophan was determined spectrophotometrically in 0.1 N NaOH following the procedure of Bencze and Schmid (1957). Threonine, serine, and amide content were obtained by extrapolation to zero time of hydrolysis while other amino acids were extrapolated to maximum recovery.

Amino-Terminal Analysis. N-terminal analysis was performed by the formation and identification of the 2,4-dinitrophenyl (DNP) (Sanger, 1945) or the phenylthiohydantoin (Edman, 1953) derivatives. The reaction of proteins (0.1–0.3 μmole) with FDNB was carried out in sodium bicarbonate solution (Porter, 1957). Peptides were allowed to react with FDNB in aqueous triethylamine (Sanger and Thompson, 1953). Hydrolysis was routinely carried out at 108° for 16 hr (overnight) in 5.7 N redistilled HCl, unless glycine or proline was suspected. The systems employed in the two-dimensional chromatography of the ether-soluble derivatives were *t*-amyl alcohol–ammonia (Fraenkel-Conrat *et al.*, 1955), and 1.5 M potassium phosphate (Levy, 1954).

The spots were eluted with 1% $NaHCO_3$, and quantitated spectrophotometrically at 360 mμ, using the extinction values reported by Fraenkel-Conrat *et al.* (1955). The acid-soluble derivatives were identified by high-voltage electrophoresis at pH 2.1.

The coupling of the phenyl isothiocyanate was accomplished by the modification of Konigsberg and Hill (1962) in *N*-ethylmorpholine. Cleavage was obtained by dissolving the PTC-protein or peptide in trifluoroacetic acid at room temperature for 2 hr. The derivative was cyclized to form the phenylthiohydantoin by heating in HCl, pH 1.0, at 80° for 1 hr (Ilse and Edman, 1963) with the exception of PTC-serine and PTC-threonine, which were cyclized in 3 N HCl at 4° overnight. Phenylthiohydantoin was quantitated by absorption at 269 mμ in ethanol. The solvent systems of Sjoquist (1960) were used in the chromatography of the phenylthiohydantoins.

Carboxyl-Terminal Analysis. The release of the carboxyl-terminal residues by bovine carboxypeptidase A or B (molar ratio substrate to enzyme 100:1) was measured at pH 8.0–9.0 at room temperature. The reaction was stopped by passing the solution through a Dowex 50 column (0.9 × 5.0 cm) which had been previously equilibrated with citrate buffer, pH 3.0. The amino acids were eluted with 1 N NH_4OH, and identified using the chromatographic system described by Richmond and Hartley (1959). The separated amino acids were quantitated by the method of Connell *et al.* (1955).

Sedimentation analysis was carried out in the Spinco Model E ultracentrifuge equipped with phase-plate Schlieren optics. Measurements were performed in a single-vector synthetic boundary cell at rotational speeds of 59,780 rpm. Radial boundary positions were measured on a microcomparator and sedimentation constants were calculated from least-squares slopes of a plot of log x *vs.* time, where x is the radial boundary position (Schachman, 1957). Buffer density was obtained by pycnometry at 20.0°. Viscosities were measured at 20.00 ± 0.01° in viscometers of the type described by Frensdorff *et al.* (1953).

Sedimentation equilibrium measurements at 0–4° were performed, utilizing the Ralyeigh interference optics, at speeds ranging from 8756 to 12,950 rpm. A double-

TABLE I: Extraction of Dogfish Pancreatic Acetone Powder.

Extractant (M)	Activities of Total Extract[d]					mg of Protein/g of Powder
	BAEE[a]	ATEE[a]	HPLA[a]	BGA[b]	CGP[b]	
H_2O	0.298	4.50	0.30	26,260	43,810	260
NaCl (1)	0.438	6.77	...	30,270	53,640	599
H_2SO_4 (0.25)	0.170	0.20	0	0	...	108
H_2SO_4 (0.125) + NaCl (1)	0.140	0	...	Trace	...	40
H_2SO_4[c] (0.01)	0.053	0.138	0.133	1,400	...	184

[a] Activity expressed in milliequivalents of base consumed per minute per gram of acetone powder. [b] Activity expressed in units per gram of acetone powder (1 unit = % hydrolysis/min). [c] pH adjusted to 3.0 with 1 N H_2SO_4. [d] In these measurements, BAEE has been used to measure activities of trypsin, ATEE to measure activities of chymotrypsin-like enzymes, HPLA and CGP to measure activities of carboxypeptidases A and B, and BGA to measure activities of carboxypeptidase B.

sector synthetic boundary cell was used and the sample layered over FC 43 (3M Chemicals) at column heights of 2.0–2.5 mm. Equilibrium was considered attained when no further shift of fringes could be detected. Hinge-point shift was followed in serial patterns. The data were plotted as the natural logarithm of the fringe number *vs.* the radial distance squared, with extrapolation to the menisci. The apparent weight-average molecular weight was calculated from the total change in concentration across the cell relative to the initial concentration according to the treatment of Lansing and Kramer (1935). Molecular weights at the menisci were evaluated from the limiting slopes of the plots according to Svedberg and Pederson (1940). The z-average molecular weight was calculated from the equation derived by Lansing and Kramer (1935).

Results

Identification and Distribution of Enzymatic Activities in Extracts of Pancreatic Acetone Powder

Aqueous extracts of pancreatic acetone powder were prepared by procedures similar to that of Wintersberger *et al.* (1962) at 4°. The acetone powder was stirred with 10 volumes of cold distilled water/g of powder. Foaming was suppressed by the addition of a drop of *n*-octyl alcohol. After 4 hr, the insoluble material was removed by centrifugation at 10,000 rpm for 20 min in a Spinco Model L preparative ultracentrifuge and the supernatant fluid was filtered through glass wool to remove debris. The pH of the clarified extract was 6.5–6.8 and activity *vs.* ATEE was <0.2% of the total potential activity. An extract prepared in this manner contained considerable amounts of nucleic acids or nucleotides as evidenced by a low absorbance ratio of 280:260 mμ (approximately 0.6). The nucleotides could be successfully removed by any one of three methods, *i.e.* (1) extensive dialysis of the extract *vs.* 0.005 M potassium phosphate buffer, pH 7.0; (2) passage of the extract over Sephadex G-50 previously equilibrated with 0.05 M ammonium acetate, pH 7.5; or (3) fractional precipitation with ammonium sulfate. Of these, the last-mentioned procedure was mostly used in this work (see below). To this end, the pH of the supernatant solution was adjusted to 7.5–8.0 by the dropwise addition of 1 N NaOH and maintained at this level throughout the subsequent procedures. DFP (1 M in isopropyl alcohol) was added to a final concentration of 1×10^{-3} M. After stirring for 1 hr, solid ammonium sulfate was added in small amounts up to 70% saturation (based on nomograph at 20°). Later (4 hr) the precipitate was removed by centrifugation at 10,000 rpm for 30 min and the supernatant solution was discarded. The precipitate was redissolved in a small volume of water, treated once again with DFP, and dialyzed *vs.* 0.005 M potassium phosphate buffer, pH 7.5. The $A_{280:260}$ ratio of the dialysate after dialysis was usually >1.65 indicative of <0.2% nucleotide. Column chromatography was subsequently performed on this solution.

Extraction of the acetone powder with 1 M NaCl instead of water served to increase more than twofold the amount of protein extracted (600 mg of protein/g of powders as compared to 260 mg when extracted with water). However, the extract was translucent and viscous (in contrast with the clear watery solution obtained after extraction with distilled water), and hence this procedure was not used for preparative purposes. Extraction of the pancreatic acetone powder with dilute acids at pH 3.0, a procedure which was introduced by Kunitz and Northrop (1935, 1936) to isolate bovine chymotrypsinogen and trypsinogen, led to a significant decrease in the yield of activatable chymotrypsinogen and trypsinogen and, as is to be expected, to a destruction of carboxypeptidase A (Table I). A similar observation has been made in the case of acid extraction of porcine pancreatic tissue (Folk and Schirmer, 1965).

Fractional precipitation with ammonium sulfate did not yield significant enrichment of any of the enzymatic activities, after activation, in the isolated fractions.

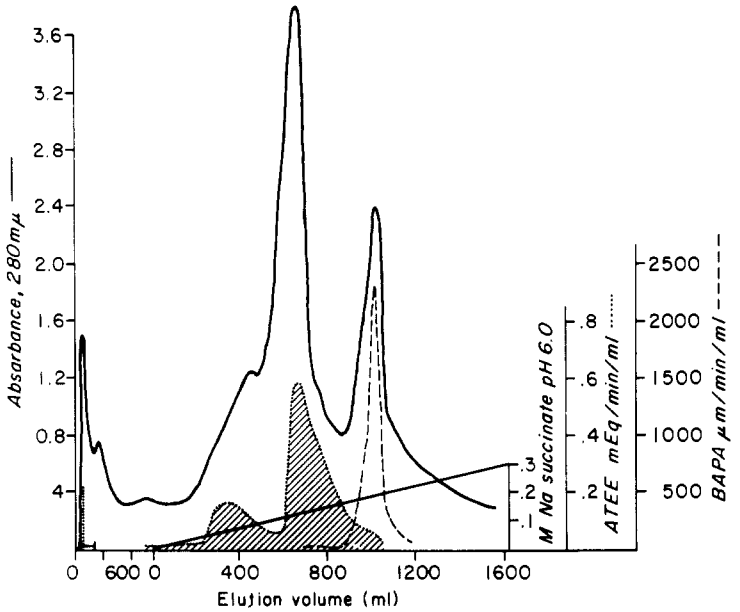

FIGURE 1: Chromatography on DEAE-Sephadex of an extract of acetone powder of dogfish pancreas. Details are given in the text. The symbols refer as follows: ———, absorbance at 280 mμ; – – – –, activity toward BAPA; . . . . ., activity toward ATEE. The protein was applied in 0.005 M potassium phosphate buffer, pH 8.0. A linear gradient was developed using 0.3 M sodium succinate buffer, pH 6.0.

Fractional precipitation was attempted between 0 and 25% saturation and thereafter in increments of 15%. The pH range from 6.4 to 9.0 was scanned in systematic fractionation experiments, but no advantage was gained by carrying out the procedure at any particular pH. Under conditions tested, enrichment of specific activity by ammonium sulfate fractionation could be achieved only at the expense of significantly decreased recovery.

Isolation and Purification of Cationic Chymotrypsinogen (A)

The starting material for the further fractionation of the aqueous extract and for the isolation of chymotrypsinogen A was a material precipitated from an aqueous extract by 70% saturation with ammonium sulfate (see above). In a typical preparation, 100 g of acetone powder was extracted and the fraction obtained by 70% ammonium sulfate saturation was collected and dialyzed *vs.* 0.005 M potassium phosphate buffer, pH 7.0. The solution was then pumped onto a column of DEAE-Sephadex A-50 (4.5 × 100 cm) which had been equilibrated with the same buffer. After appearance of the breakthrough peak, the remaining proteins were eluted from the column by linear gradient developed by use of 0.3 M sodium succinate buffer, pH 6.0, as shown in Figure 1. The breakthrough peak, presumably containing unabsorbed cationic proteins, represented approximately 15–20% of the total absorbance at 280 mμ applied to the column. Upon activation with bovine trypsin, only activity toward ATEE could be identified, representing 20% of the total potential ATEE hydrolyzing activity present in the original extract. The effluent fractions resulting from chromatography on DEAE-Sephadex were tested for endopeptidase activity toward ATEE and BAPA, respectively, with the results shown in Figure 1. These two activities, characteristic in general of chymotrypsin and trypsin, respectively, were clearly separated from each other, the tryptic activity appearing last, suggesting that it was associated with a protein of anionic character. In analogy with bovine pancreatic extract (Keller *et al.*, 1956, 1958) the ATEE-hydrolyzing activity of the anionic protein components is probably associated with an anionic form of chymotrypsin. Chromatographic purification of the unabsorbed cationic proteins on CM-cellulose at pH 4.7–5.0 proved to be superior to separation at pH 7.0–8.0 and was therefore applied in the subsequent isolation procedure.

The breakthrough fraction was rechromatographed after lyophilization by dissolving the material first in 100 ml of 0.1 M sodium succinate buffer, pH 5.0, and dialyzing overnight *vs.* 12 l. of 0.005 M sodium succinate buffer, pH 5.0. The solution was then applied to a column of CM-cellulose (1.4 × 40 cm) which had been equilibrated with the same buffer. The column was washed free of unabsorbed protein and a linear NaCl gradient (0–0.2 M) in the same buffer developed (Figure 2). The crude protein fractions were then rechromatographed in the same manner (Figure 3) except that the protein was not previously lyophilized but the solution was pumped directly onto the column after dialysis *vs.* 0.005 M sodium succinate. Rechromatography (Figure 3) resulted in improvement of the specific activities of the fractions constituting the shoulder of the peak but not of those of the peak itself as indicated by the relative and specific activities given in Figure 3. The yield of

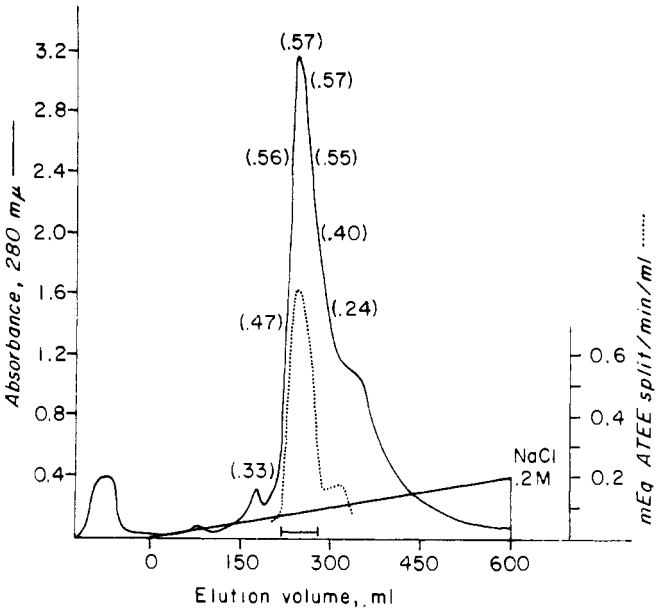

FIGURE 2: Chromatography on CM-cellulose of the cationic fraction obtained from the chromatography of the extract of dogfish pancreatic acetone powder upon DEAE-Sephadex. Details are given in the text. ———, absorbance at 280 mμ; . . . . ., activity toward ATEE after tryptic activation. The protein was applied in 0.005 M sodium succinate buffer, pH 5.0, and a linear gradient then developed with sodium chloride. Bracketed fractions were pooled and concentrated. The numbers in parentheses are specific activities after activation by trypsin.

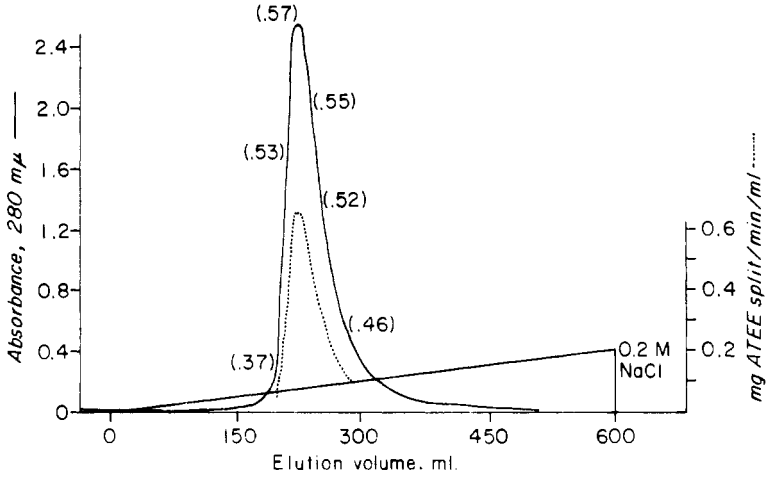

FIGURE 3: Rechromatography upon CM-cellulose of the cationic dogfish chymotrypsinogen obtained from previous chromatography of the cationic fraction. For further details see the text and the legend to Figure 2.

cationic chymotrypsinogen after the final chromatography was 75–100 mg/100 g of acetone powder.

Properties of Chymotrypsinogen A

The characterization of cationic chymotrypsinogen was carried out on pooled preparations which upon activation with trypsin gave nearly constant specific activities (0.52–0.57).

Sedimentation Analysis. Measurements of the sedimentation velocity in Tris–HCl buffers, pH 8.0, ionic strength 0.01, containing 0.1 M NaCl, at 4–8° showed a marked increase of $s_{20,w}$ with increasing protein concentration (Figure 4). The refractive index gradient patterns revealed slight skewing. Behavior of this type, which has also been observed with bovine α-chymotrypsin (Schwert, 1949), is usually attributed to protein–protein interaction with the formation of high molecular weight polymers. In contrast, at pH 3.0 (glycine–HCl buffer, ionic strength 0.1 at 20°), the patterns appeared symmetrical and the calculated sedimentation coefficient

TABLE II: Molecular Weights Obtained by Sedimentation Equilibrium Analysis.

Protein (mg/ml)	pH	Buffer (M)	M_w[a] ($\times 10^{-3}$)	M_z[b] ($\times 10^{-3}$)	M_w/M_z
3.52	4.96	NaOAc (0.1)	30.0	39.2	0.77
5.00	5.05	Same	27.7	37.0	0.75
5.52	5.05	Same	29.1	50.3	0.58
3.14	4.96	Same	23.2	25.0	0.93
5.71	5.0	Same	24.8	26.8	0.92
0.97	4.96	Same	19.6	...	...
4.87	7.50	KPO_4 $\Gamma/2$ = 0.1	35.5	38.7	0.92
2.84	3.0	Glycine–HCl $\Gamma/2$ = 0.1	25.4	...	...

[a] M_w = apparent weight-average molecular weight. [b] M_z = z-average molecular weight.

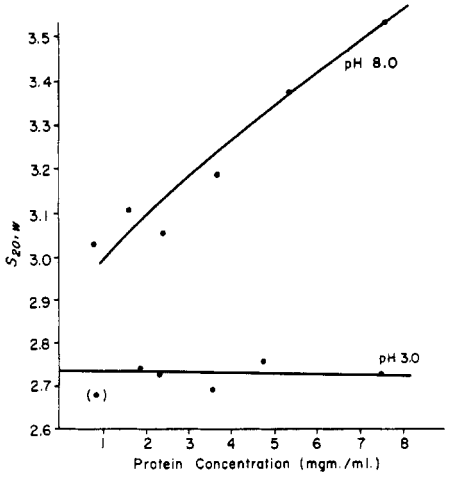

FIGURE 4: Dependence of the sedimentation velocity of cationic dogfish chymotrypsinogen upon protein concentration. Analyses were performed in a single-section synthetic boundary cell at 59,780 rpm. Tris–HCl buffer ($\Gamma/2$ = 0.01) containing 0.1 M sodium chloride was used for the measurements at pH 8.0. Glycine–HCl buffer ($\Gamma/2$ = 0.1) was employed at pH 3.0. See text for further details.

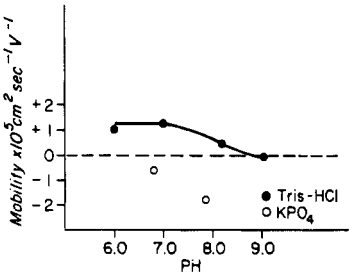

FIGURE 5: Dependence of electrophoretic mobility of cationic dogfish chymotrypsinogen upon pH. Tris–HCl buffers of ionic strength of 0.1 were used. The potassium phosphate buffers were 0.1 M.

was linear with protein concentration (Figure 4). If the point of the lowest protein concentration (0.84 mg) is omitted because of the uncertainty of calculation, the remaining points follow a straight line with a slightly negative slope (−0.004) which extrapolates to $s_{20,w}$ = 2.73 S.

Sedimentation Equilibrium and Molecular Weight. The results of these calculations are summarized in Table II. The plots on which these data are based varied from linear to upward concavity, an indication of heterogeneity. With an assumed partial specific volume of 0.735 ml/g, the apparent weight-average molecular weight was approximately 24,000–25,000.

Absorbancy Index. The extinction coefficient for a 1% solution at 280 mμ was obtained by relating optical density to the protein concentration. When the latter was determined in the ultracentrifuge, using refractometric methods, a value of 21.7 was calculated. Calculations based on the anhydrous residue weights obtained from amino acid analysis yielded a value of 21.4.

Electrophoresis. Free-boundary electrophoresis of the protein was performed in the Spinco Model H electrophoresis apparatus at 1° for periods up to 625 min at protein concentrations of 5–7.5 mg/ml. At least 95% of the protein migrated as a single peak. In univalent buffers (ionic strength 0.2), the isoelectric point of the protein was pH 8.7 (Figure 5). In phosphate buffers, however, the isoelectric point shifted approximately to pH 6.5, indicative of phosphate binding.

Amino Acid Composition. The results summarized in Table III represent the total recovery based on internal standards. Of all the values reported, that of tryptophan is probably the least reliable. Brown (1963) has observed the failure of the spectrophotometric determination by the method of Bencze and Schmid (1957) in those cases in which tryptophan content exceeds that of tyrosine. The minimum molecular weight

TABLE III: Amino Acid Composition of Dogfish Chymotrypsinogen A.

	Residues/ 10^5 g of Protein	g Residue/10^5 g of Protein	Min Mol Wt	Residues/ 24,500 g of Protein	Nearest Integer/ 24,500 g	Nearest Integer × Min Mol Wt	Integral No. × Mol Wt of Residue
Alanine	93.3	6,632	1,062	22.9	23	24,403	1,635
Arginine	28.2	4,409	3,511	6.92	7	24,577	1,093
Aspartic acid	83.3	9,581	1,190	20.4	20	23,800	2,302
Glutamic acid	52.7	6,798	1,883	12.9	13	24,479	1,678
Glycine	87.4	4,988	1,134	21.4	21	23,814	1,199
Histidine	16.3	2,233	6,089	3.99	4	24,356	549
Isoleucine	40.2	4,544	2,490	9.84	10	24,900	1,132
Leucine	45.8	5,186	2,162	11.2	11	23,782	1,245
Lysine	41.2	5,281	2,406	10.1	10	24,060	1,282
Methionine	14.4	1,883	6,690	3.5	4	27,840	525
Phenylalanine	12.5	1,841	7,925	3.0	3	23,775	442
Proline	55.9	5,432	1,800	13.7	14	25,200	1,360
Serine	87.2	7,592	1,137	21.4	21	23,877	1,829
Threonine	58.9	5,954	1,692	14.4	14	23,688	1,415
Tyrosine	22.8	3,721	4,347	5.6	6	26,082	979
Valine	92.8	9,201	1,067	22.7	23	24,451	2,281
Amide	81.9	1,393	...	20.1	20	...	340
Half-cystine[a]	34.3	3,538	...	8.4	8	...	825
Tryptophan[b]	47.9	8,916	...	11.7	12	...	2,234
		99,123			224	24,568 ± 724	24,345

[a] Determined as cysteic acid after performic acid oxidation (Moore, 1963). [b] Determined spectrophotometrically according to Bencze and Schmid (1957).

calculated from these data is 24,568 ± 724 which agrees with the value obtained by sedimentation analysis.

Amino-Terminal Residues. Reaction of the chymotrypsinogen with FDNB in sodium bicarbonate solutions followed by acid hydrolysis yielded on two-dimensional chromatography of the ether-soluble derivatives, bis-*N*-DNP-cystine, probably arising from disulfide interchange. A trace of DNP-cysteic acid was identified upon high-voltage electrophoresis of the aqueous phase at pH 2, 2000 v for 70 min. The small quantities of DNP-serine and DNP-aspartic or glutamic acid are considered to be insignificant impurities. If the DNP-protein was first subjected to performic acid oxidation at 0° for 45 min before hydrolysis (Bettelheim, 1955) bis-*N*-DNP-cystine was no longer identifiable in the ether phase, while the recovery of DNP-cysteic acid increased to a stoichiometrically significant level of approximately 1 mole/mole of protein. The uncorrected recoveries are given in Table IV.

TABLE IV: DNP-Amino Acids Isolated from DNP-Dogfish Chymotrypsinogen.[a]

Phase	Compd	Un-oxi-dized	Oxi-dized
Ether	Bis-*N*-DNP-cystine	0.036	0
	N-DNP-serine	0.031	0.031
	N-DNP-Asp/Glu	0.044	0.063
Aqueous	ε-*N*-DNP-lysine	3.09	4.08
	N-DNP-cysteic	0.088	0.513

[a] Recoveries are reported in moles/24,500 g of protein, uncorrected. Oxidation was performed according to Bettelheim (1955).

Activation of Chymotrypsinogen. The zymogen could be rapidly activated by bovine trypsin in the pH range of 6.5–8.5 at 0°. At pH 7.5–8.0, 0°, activation of dogfish chymotrypsinogen (1×10^{-5} M) by trypsin (1×10^{-6} M) reached completion within 2 hr. At constant zymogen concentration (3.4×10^{-6} M) in a 0.01 M Tris–HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.01 M $CaCl_2$, the rate of activation followed first-order kinetics when the zymogen–trypsin ratio was varied over the range of 10:1–1000:1 (Figure 6). Except for the highest concentration of trypsin investigated, the rate was proportional to trypsin concentration. The presence of Ca^{2+} had no measureable effect on activation except at the lowest trypsin concentration where, presumably, it served to stabilize this enzyme. The time course of

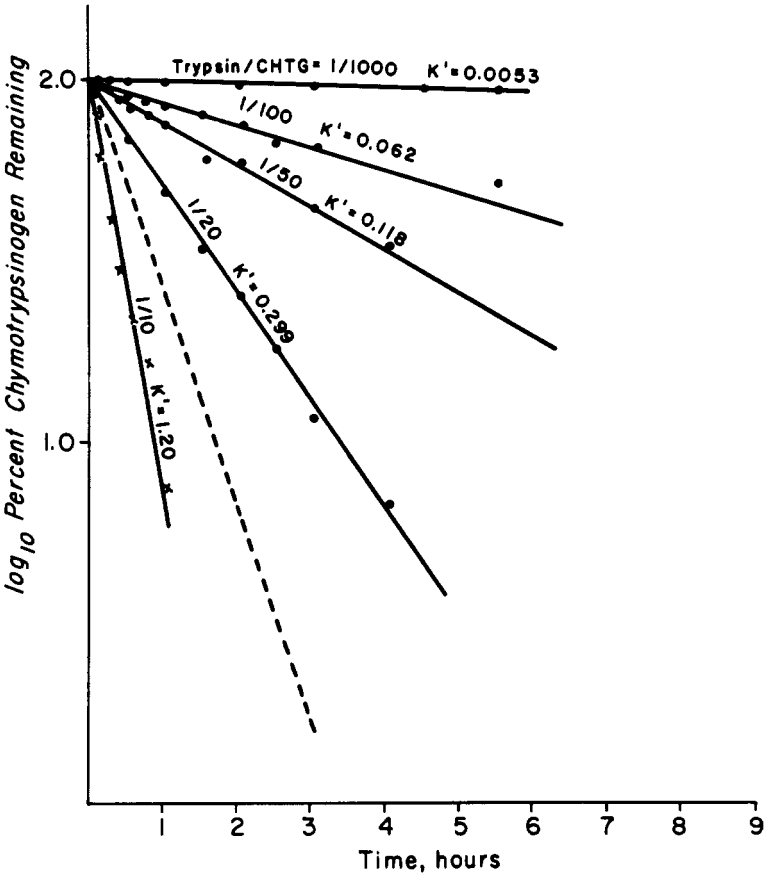

FIGURE 6: Effect of trypsin concentration on the rate of activation of cationic dogfish chymotrypsinogen. The zymogen concentration was 3.4×10^{-6} M. The buffer system was Tris–HCl (ionic strength = 0.01), pH 8.0, containing 0.1 M NaCl and 0.01 M $CaCl_2$. Activation performed at 0°. The dotted line has been calculated for a 1:10 ratio from the slopes obtained at lower trypsin–zymogen weight ratios.

activation observed in the presence of 0.1 M β-phenylpropionate, a chymotrypsin inhibitor, was indistinguishable from that observed in the absence of this inhibitor (Figure 7). The same time course of activation was observed with succinylated bovine trypsin as with native trypsin and the presence of β-phenylpropionate produced no change in the course of activation by the succinylated enzyme. In no instance was any intermediate of higher specific activity observed in the process of "rapid activation" as had been observed in the activation of bovine chymotrypsinogen A (Jacobsen, 1947; Bettelheim and Neurath, 1955). The maximum activity obtained during activation in the presence of high concentrations of trypsin (zymogen–trypsin ratio, 10:1) decreased only 5–7% over a 24-hr period at 0°. Bovine α-chymotrypsin, as to be expected, was incapable of activating the dogfish chymotrypsinogen.

Chemical Events Associated with the Activation of Chymotrypsinogen. The tryptic activation of dogfish chymotrypsinogen is associated with the appearance of new amino-terminal residues which have been identified by the following procedure. A solution containing 0.21 μmole (5.25 mg/ml) of chymotrypsinogen was incubated at 0° in 0.1 M potassium phosphate buffer, pH

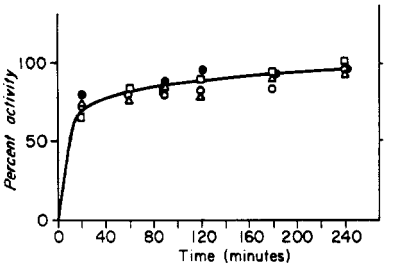

FIGURE 7: The activation of cationic dogfish chymotrypsinogen at 0° by trypsin and succinyl trypsin in the presence and absence of 0.1 M β-phenylpropionate (β-pp). O, trypsin, △, trypsin plus β-pp; □, succinyl trypsin, ●, succinyl trypsin plus β-pp.

8, with bovine trypsin (weight ratio, 50:1). At suitable time intervals, aliquots were removed and pipetted into 0.1-ml buffer containing 0.01 mg of STI. These solutions were then assayed for activity using ATEE as substrate. At time 0, 60, and 180 min, another aliquot containing 0.21 μmole of chymotrypsinogen was removed and pipetted into a buffer solution containing

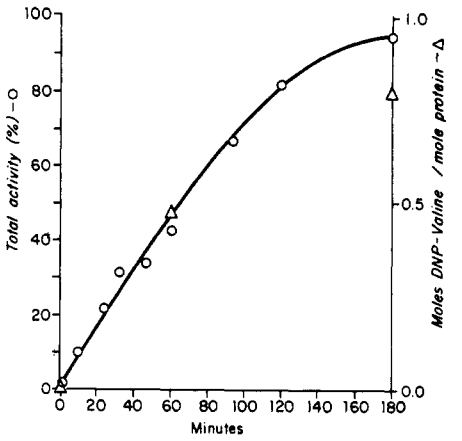

FIGURE 8: The appearance of N-terminal valine during the activation of cationic dogfish chymotrypsinogen by trypsin. Mole ratio of chymotrypsinogen–trypsin, 50:1. Esterase activity represented by circles, mole fraction of valine by triangles.

0.02 mg of STI and 10^{-2} M DFP. After 1 hr, these solutions were frozen, lyophilized, and analyzed for amino-terminal residues with FDNB as described above. Only DNP-valine could be found in the ether phase, the uncorrected recoveries being 0.23 and 0.38 μmole/μmole of chymotrypsinogen after 60 and 180 min of activation, respectively. The correlation of the corrected yields of this new end group with the appearance of enzymatic activity is shown in Figure 8. The identity of DNP-valine was further confirmed by thin layer chromatography on silica gel using a solvent system of benzene–pyridine–acetic acid, 80:20:2 (Brenner *et al.*, 1961). No other DNP-amino acid could be found in significant quantities in the ether phase, and only ε-DNP-lysine was found in the aqueous phase.

The rapid activation of bovine chymotrypsinogen A is accompanied by the tryptic cleavage of an arginyl–isoleucyl bond to form π-chymotrypsin and is followed by the release of a serylarginine dipeptide by autolytic attack, the second step leading to the formation of δ-chymotrypsin (Bettelheim and Neurath, 1955; Rovery *et al.*, 1955a,b). Experiments to establish the release of a peptide in the activation of dogfish chymotrypsinogen under the conditions described herein gave negative results. In these experiments, activation was carried out under the conditions just described both in the presence and absence of 0.1 M β-phenylpropionate. The possible release of free amino acids or peptides was determined by the procedure of Gladner and Neurath (1953) using columns of Dowex 50-X4 (0.7 × 6 cm, Bio-Rad Laboratories, Lot 27OB-381) in the hydrogen form to absorb these products from acidified solutions (pH 2.5–3.0) of the activation mixture. After elution with 1 N ammonia the eluates were examined after high-voltage electrophoresis at 2000 v for 70 min at pH 6.5 by ninhydrin staining of the electropherograms. Staining with Sakaguchi reagent also gave negative results in all cases. The inability to identify any arginine or arginyl peptides in the activation mixtures precludes a mechanism of activation similar to that of the bovine zymogen.

The protein which was not absorbed by the Dowex 50-X4 column represented dogfish DIP-chymotrypsin and was examined for the presence of basic carboxyl-terminal residues (arginine or lysine) by digestion with dogfish carboxypeptidase B prepared as described in a forthcoming paper (J. W. Prahl and H. Neurath, in preparation). To this end, the lyophilized protein was dissolved in 0.1 M potassium phosphate buffer, pH 8, containing 10^{-2} M DFP, and incubated with carboxypeptidase B (substrate–enzyme, weight ratio of 100:1) for 2 hr at pH 7.8–8.0 at room temperature. The free amino acids released during this process were converted to the corresponding DNP derivatives by adding solid sodium bicarbonate and FDNB. After standing overnight, the excess FDNB was extracted with peroxide-free ether, the reaction mixture acidified to pH 1.0 with 12 N HCl, and any free DNP-amino acids separated from insoluble DNP-protein by centrifugation. The ether-extractable phase was examined for bis-DNP-lysine which was found only in insignificant quantities and the aqueous phase was examined for DNP-arginine by paper chromatography in *n*-butyl alcohol–acetic acid–water (4:1:5). The results shown in Table V indicate that the activation of the dogfish zymogen involves the cleavage of an arginyl–valyl peptide bond by trypsin.

As is to be expected, examination of the amino-terminal residue of DNP-DIP-chymotrypsin, after digestion with carboxypeptidase B, yielded DNP-valine as a major product. Unexpectedly, however, DNP-glycine was also obtained in amounts of approximately one-third of that of DNP-valine (uncorrected) (Table V).

Isolation and Characterization of Chymotrypsin

Dogfish chymotrypsin can be prepared from activated chymotrypsinogen by chromatography on CM-cellulose. To this end, succinylated bovine trypsin was used for activation of chymotrypsinogen since native bovine trypsin could not be successfully resolved by chromatography from chymotrypsin whereas the succinylated bovine trypsin was not absorbed by CM-cellulose. Activation was performed in 0.1 M potassium phosphate buffer, pH 8.0, in the presence of 0.1 M β-phenylpropionate at 0°. The zymogen concentrations were 0.5% or greater and succinyl trypsin was added in a ratio of 1:30. When a plateau of constant maximum activity was reached, a twofold excess of STI (with respect to succinyl trypsin) was added, the pH adjusted to 4.7–5.0, and the solution dialyzed *vs.* 0.005 M sodium succinate buffer, pH 5. This material was then subjected to chromatography on CM-cellulose employing a linear gradient of 0–0.4 M NaCl as shown in Figure 9. Pooled fractions of maximum constant specific activity toward ATEE were then used for further characterization of the enzyme as described below. Due to the low yield it has not yet been possible to prepare the active enzyme by crystallization procedures similar to those em-

TABLE V: Amino- and Carboxyl-Terminal Amino Acid Residues of Activated Dogfish Chymotrypsinogen.[a]

Activation	% Max Activity	Carboxyl-Terminal Residues[c]		Amino-Terminal Residues[b]			
		DNP-arginine[d]	Bis-DNP-lysine[e]	DNP-Val	DNP-Glu-Asp	DNP-Gly	Bis-DNP-Cys
2 hr − β-PP	86	0.58	0.01	0.30	0.05	0.11	0.04
2 hr + β-PP	62	0.47	0.01	0.24	0.04	0.10	0.03
40 hr + β-PP	73	0.63	0.07	0.27	0.02	0.07	0.03

[a] Moles/24,500 g of protein (uncorrected). [b] Determined after acid hydrolysis of the DNP-protein. For details see the text. Also identified were traces of DNP-serine, -alanine, -threonine, and leucine or isoleucine. [c] DNP-amino acids isolated on hydrolysis of DNP-chymotrypsin after digestion with carboxypeptidase B (see the text). [d] Separated by paper chromatography in *n*-butyl alcohol–acetic acid–water (4:1:5). [e] Ether phase examined only for bis-DNP-lysine.

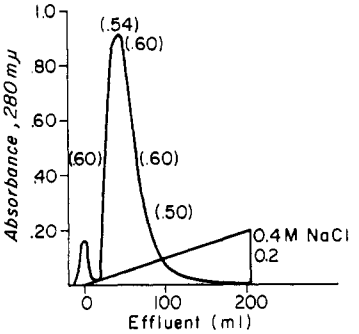

FIGURE 9: Chromatography on CM-cellulose of cationic dogfish chymotrypsin obtained by activation of the zymogen with succinyl trypsin. See text for details.

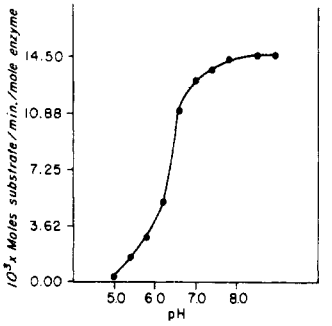

FIGURE 10: pH dependence of the esterase activity of dogfish chymotrypsin toward ATEE. For details see the text.

ployed for the corresponding bovine enzyme (Northrop *et al.*, 1948).

Enzymatic Activity

When assayed toward ATEE (0.01 M) as substrate at pH 7.9–8.0 at 25°, the specific activity of the best preparations of dogfish chymotrypsin was approximately 0.58 mequiv of base consumed/min/mg of protein, which is approximately 17% higher than the specific activity reported for the bovine enzyme under the same conditions (Cunningham and Brown, 1956). As with bovine chymotrypsins A and B, the dogfish enzyme is capable of hydrolyzing also the synthetic substrate acetyl-L-tryptophan ethyl ester. Keller *et al.* (1958) had observed that the presence of 30% methanol in the assay mixture produced a marked depression of the specific activity of bovine chymotrypsin B toward this substrate, and Guy (1961) subsequently extended this observation to distinguish between bovine chymotrypsins A and B, respectively. Comparison of dogfish chymotrypsin with the bovine enzymes on this basis is presented in Table VI. The data suggest that the dogfish enzyme occupies a position intermediate between the bovine enzymes A and B in this regard.

Dogfish chymotrypsin exhibits maximal activity toward ATEE in the pH range of 7.5–8.0. The pH dependence (Figure 10) in the acid range showed an inflection at approximately pH 6.35–6.50, a behavior which is also revealed by bovine chymotrypsin A (Cunningham and Brown, 1956).

Since the body temperature of the dogfish is considerably lower than that of the bovine, it was of in-

TABLE VI: Comparison of Esterase Activity of Chymotrypsins toward ATEE and ATrpEE in the Presence of Methanol.

Chymotrypsins	ATEE			ATryEE	
	0%	5%	30%	5%	30%
Bovine A	48	48	13	15	13
Bovine B	43	36	9.8	11	0.8
Dogfish A	59	45	9.6	30	16

[a] Expressed in milliequivalents of base uptake per minute per milligram of protein × 10^{-2}. Methanol concentrations are expressed in volume per cent.

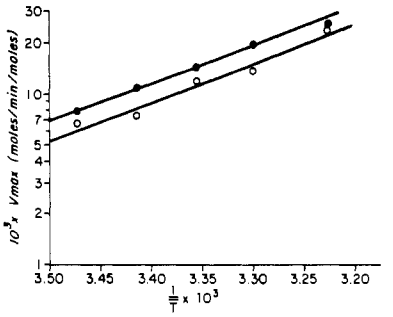

FIGURE 11: Temperature dependence of the esterase activity of cationic dogfish chymotrypsin. The energy of activation (E_a) was calculated from the Arrhenius equation. N, dogfish A, E_a = 13,600 cal/mole; C, bovine A, E_a = 13,000 cal/mole.

terest to determine the temperature dependence of the activity of this enzyme in the range of 15–37°. In these experiments, ATEE was the substrate, at an initial concentration of 0.01 M. All measurements were carried out in the presence of 0.01 M potassium phosphate buffer, pH 8.0, containing 0.1 M KCl. The initial measured velocity v was considered to approximate k_3, the rate of decomposition of the Michaelis–Menten complex. An Arrhenius plot of the data is shown in Figure 11 for both dogfish chymotrypsin and bovine chymotrypsin A. From these data, an activation energy of 13.6 kcal/mole was calculated for the dogfish enzyme as compared to 13.0 for the bovine. The latter compares favorably with the value of 11.5 reported by Snoke and Neurath (1950) in the presence of 30% methanol and of 11 ± 2 kcal/mole reported by Cunningham and Brown (1956) who used acetyl-L-tryptophan ethyl ester as substrate.

Dogfish chymotrypsin, like the bovine enzyme, is inhibited by DFP, PMSF, β-phenylpropionate, indole, and 3-indolepropionate. No inhibition could be demonstrated by *p*-hydroxymercuribenzoate (10^{-3} M), by EDTA (10^{-2} M), cysteine (10^{-2} M), or Zn, Mg, or Ca (all at 10^{-1} M).

The competitive inhibition by β-phenylpropionate, indole, and 3-indolepropionate was determined using ATEE as substrate (0.1 and 0.05 M) in 0.01 M potassium phosphate buffer, pH 8.0. The inhibitor was varied from 1 to 12 × 10^{-3} M and the ionic strength was adjusted to 0.2 by the addition of KCl. Eadie (1942) plots of the data yielded K_i values of 1.62 × 10^{-2} M, 2.0 × 10^{-3} M, and 8.3 × 10^{-4} M, respectively, for β-phenylpropionate, indole, and 3-indolepropionate. The corresponding values for the bovine enzyme are 0.45 × 10^{-2} M (Neurath and Schwert, 1950), 0.8 × 10^{-3} M (Wallace *et al.*, 1963), and 1.3 × 10^{-4} M (Huang and Niemann, 1952). All measurements were performed at 25°. The specificity of dogfish chymotrypsin was compared to the bovine using glucagon as a protein substrate. Enenkel and Smillie (1963) have demonstrated the marked difference in specificity of bovine chymotrypsins A and B, respectively, toward this protein. In a qualitative manner, the action of the dogfish enzyme was compared to that of the bovine by examination of "fingerprints" of the digest. The fewest number of peptides was found when digestion was carried out with bovine chymotrypsin A and the largest number with the dogfish enzyme. Examination of the N-terminal residues in the digest indicated that all such residues obtained by digestion with bovine chymotrypsin A and B were also found with the dogfish enzymes (DNP-leucine, valine, aspartic acid, threonine, serine, and methionine). A more detailed investigation of the nature of the bonds being split has not yet been carried out.

Discussion

The presence of zymogens of proteolytic enzymes having the characteristics of chymotrypsins A and B, trypsin, and carboxypeptidases A and B was first demonstrated in the pancreatic juice of the bovine by Keller *et al.* (1958), and confirmed by Greene *et al.* (1963). By similar techniques, the pancreatic juice of the pig, dog (Marchis-Mouren, 1959), and rat (Marchis-Mouren *et al.*, 1963) have been shown to contain the same general spectrum of zymogens with some variations which will be considered later in this discussion. More recently, extracts of avian pancreas (chicken and turkey) (Ryan, 1965; Ryan *et al.*, 1965) have also been found to contain enzymatic activities characteristic of chymotrypsin and trypsin, respectively. The present study has extended these observations to the pancreatic proteins of a phylogenetically distant species, *Squalus acanthias*, commonly known as the spiny Pacific dogfish. In this case an aqueous extract of an acetone powder of the pancreas dissected from the living animal has served as source of proteins rather than the pancreatic juice itself. Judging from the results with the bovine system, the findings are probably unaffected by the different starting material. As in the case of the bovine, the anionic and cationic components could be easily separated from each other by passage through a suitable ion exchange absorbent. In contrast to the mammalian tissues, however, the dogfish appears to possess only chymotrypsinogen among the cationic components, whereas trypsinogen, as in the case of the rat, appeared in the anionic fraction and seems to be acidic rather than basic in character.

Bovine pancreatic juice contains several zymogens which, upon activation, give rise to enzymes which hydrolyze certain synthetic substrates for chymotrypsin. Besides the cationic chymotrypsinogen A, chymotrypsinogen B having anionic character has been described by Laskowski and co-workers (Keith *et al.*, 1947) and has been recently more fully characterized (Enenkel and Smillie, 1963; Hartley *et al.*, 1965). In addition, Brown *et al.* (1963) have shown that the precursor of carboxypeptidase A, procarboxypeptidase A, is an aggregate of three subunits and one of these, subunit II, also yields a chymotrypsin-like enzyme on activation. In the porcine pancreatic juice, proteins analogous to cationic and anionic chymotrypsinogens have been found by Marchis-Mouren (1959) and Uriel

TABLE VII: Comparison of Chymotrypsinogens of Various Sources.

	Bovine A	Porcine A	Dogfish (A)	Fraction II	Bovine B	Porcine C
Isoelectric point	9.1	7.2	8.7	Anionic	5.2	Anionic
Molecular weight	25,761	22,700	24,500	25,000	26,000	31,800
N-terminal residue	←----------Half-cystine----------→					
C-terminal residue	$AspNH_2$				$AspNH_2$	
Bond being cleaved during activation	Arg-Ile	Arg-Ile	Arg-Val	?-Val	Arg-Ile	Arg-Val

and Avrameas (1965). Recently Folk and Schirmer (1965) have described a porcine anionic chymotrypsinogen C which is not a counterpart of the bovine chymotrypsinogen B. While the enzymes derived from all of these zymogens are active toward peptide and ester substrates wherein an aromatic residue contributes the carbonyl function, some differences in the specificity have been noted when tested toward other substrates. Of those investigated, the porcine and bovine cationic chymotrypsins demonstrated the strictest specificity, the bovine anionic chymotrypsin an intermediate specificity, and the porcine anionic chymotrypsin and dogfish cationic chymotrypsin the broadest specificity, when tested on glucagon as substrate (Enenkel and Smillie, 1963; Folk and Schirmer, 1965; Folk and Cole, 1965).

Except for porcine chymotrypsinogen C, all chymotrypsinogens have nearly the same molecular weight and all of them, whether they are cationic or anionic, contain half-cystine as the amino-terminal residue (Table VII). Where known, the carboxyl-terminal residue is asparagine. The isoelectric point of the cationic dogfish chymotrypsinogen is intermediate between that of the cationic bovine and porcine zymogens.

Comparison of the amino acid composition of the chymotrypsinogens (Table VIII) shows that the best agreement exists among bovine chymotrypsinogens A and B and porcine chymotrypsinogen A. The dogfish zymogen demonstrates some similarities to this group, but the resemblence in amino acid composition to that of the porcine chymotrypsinogen C and fraction II of bovine procarboxypeptidase A is perhaps more striking. In the porcine chymotrypsinogen A and bovine chymotrypinogens A and B, 10 half-cystine residues have been identified, all of which are present as intrachain disulfide bonds (Rovery *et al.*, 1960; Hartley, 1964; and Smillie and Hartley, 1965). Only eight half-cystine residues have been reported in the dogfish zymogen and in fraction II of bovine procarboxypeptidase A (Brown *et al.*, 1963). Although similarities in amino acid composition are not necessarily indications of similarities in sequence (Matsubara and Smith, 1963), in the case of bovine trypsinogen and chymotrypsinogen A, this relationship is clearly evident (Hofmann, 1964; Walsh and Neurath, 1964). For this reason, it may be suggested that the similarity in amino acid composition among the five chymotrypsinogens may reflect a considerable measure of homology in primary structure.

All five chymotrypsinogens listed in Table VII are capable of being activated by trypsin and in all but fraction II of bovine procarboxypeptidase A, tryptic activation involves the hydrolysis of a peptide bond containing the carboxyl group of an arginine residue. In the activation of bovine chymotrypsinogen A (Rovery and Desnuelle, 1954), bovine chymotrypsinogen B (Rovery *et al.*, 1960), and porcine chymotrypsinogen A (Rovery, 1964), an arginyl–isoleucyl bond is cleaved, while in the activation of dogfish cationic chymotrypsinogen and porcine chymotrypsinogen C (Folk and Schirmer, 1965), an arginyl–valyl bond is broken. Brown *et al.* (1963) were able to identify the appearance of a new amino-terminal valine residue during activation of fraction II of bovine procarboxypeptidase A by bovine trypsin but the identity of the corresponding carboxyl-terminal residue was not reported.

The "rapid" activation of bovine chymotrypsinogen A is characterized by the cleavage of a second peptide bond when π-chymotrypsin is converted to the δ form, resulting in the liberation of the dipeptide serylarginine (Bettelheim and Neurath, 1955; Dreyer and Neurath, 1955). During the activation of bovine chymotrypsinogen B which contains an alanyl residue in place of the seryl (Guy *et al.*, 1966) the second peptide bond is resistant to hydrolysis. Porcine chymotrypsinogen A behaves in this regard much like bovine chymotrypsinogen B (Rovery, 1964) despite the fact that it possesses the same sequence as the bovine cationic zymogen in the region affected by activation.[2] Activation of cationic dogfish chymotrypsinogen, even if extended much beyond the time required for maximum activity to appear, likewise fails to give rise to any peptide.

Dogfish chymotrypsin, obtained by tryptic activation of chymotrypsinogen, shows sufficient similarities to the bovine enzyme to justify the conclusion that, in fact, it represents a truly homologous form. This conclusion is based not only on the substrate specificity as determined in this investigation but also on irreversible inhibition of the enzyme by DFP and by PMSF. While its classification as a "serine" enzyme seems to be

[2] This anomaly can possibly be explained as being due to a steric effect of a proline residue occurring several residues removed on the amino-terminal side of this area (Neurath, 1964).

TABLE VIII: Amino Acid Composition of Known Chymotrypsinogens.

	Bovine[a] A	Porcine[b] A	Dogfish A	Fraction II[c] PCP-A S_6	Bovine[d] B	Porcine[e] C
Alanine	22	20–21	22	15	23–24	14
Arginine	4	5	7	8	5	7
Aspartic acid	23	18	20	24	20	25
Glutamic acid	16	12	13	21	19–20	22
Glycine	20	19	21	21	23	24
Histidine	2	2	4	5	2	5
Isoleucine	10	9	10	12	8–9	12
Leucine	19	17	11	20	19	19
Lysine	14	10	10	7	11	7
Methionine	2	2	4	1	4	1
Phenylalanine	6	5	3	7	7	5
Proline	9	12–13	13	11	14–15	12
Serine	30	21–22	21	13	21–22	20
Threonine	23	19	14	16	22–23	13
Tryptophan	8	7	12	13	8	11
Tyrosine	4	4	6	6	3–4	6
Valine	23	22	22	18	24	18
Half-cystine	10	10	8	8	10	8
Amide	24	14	20	..	18	..

[a] Hartley (1964). [b] Rovery *et al.* (1960). [c] Brown *et al.* (1963). [d] Guy *et al.* (1966). [e] Folk and Schirmer (1965) recalculated on the basis of 25,000 mol wt for purposes of comparison.

beyond doubt, the contribution of a histidine moiety to the active center can only be implied by the pH dependence of enzymatic activity which is clearly analogous to that of the bovine. Inhibition by "histidine reagents" such as TPCK (Schoellmann and Shaw, 1963) remains to be established.

From the viewpoint of evolution of the species, it is of interest that in all species thus far examined, an anionic form of chymotrypsinogen appears to be present; however, two species have been found thus far in which a cationic form of chymotrypsinogen definitely does not exist, *i.e.*, the rat and the dog. Taken together these observations suggest the possibility that the "primeval" chymotrypsinogen resembled more closely the contemporary anionic forms, the cationic forms in the fish and higher mammals having evolved independently from progeny of the anionic form. If this should be so, then a far closer homology is to be expected from a comparison of the properties and amino acid sequence of the various anionic species than the cationic ones. The sporadic lack of the cationic form among some species of mammals does not support the alternate possibility, namely, the formation of cationic and anionic lines early in the evolution of the system and maintained independently during development.

A similar picture of complexity appears to be emerging in the evolution of the present mammalian trypsinogens. The trypsinogens of the bovine, porcine, canine, ovine, and equine have been identified, and established as cationic proteins. In the rat and the dogfish, however, the zymogen has been found to be associated with the anionic components of the pancreatic proteins. Croston and Halver (1961) reported the existence of endopeptidases of basic specificity in both the cationic and anionic components of the pancreatic proteins of the chinook salmon (*Onchorhynchus tschawytscha*). This observation is of unusual interest since the salmonoides represent a phylogenetically more advanced form of vertebrates than does the elasmobranch. In the case of the dogfish, the anionic character of trypsinogen has been verified both by its chromatographic behavior and its mobility on free-boundary electrophoresis. Although by no means pure, the protein shows a minimum solubility in the pH range of 4.0–5.0, suggesting that its isoelectric point is in that range. Low yields and instability have precluded more detailed characterization of dogfish trypsinogen, but attempts are being continued to isolate this protein in pure form in order to compare its molecular properties with those of the corresponding proteins of the bovine and porcine pancreas.

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