Acknowledgment

Computer programs used in addition to MULTAN were written by Dr. H. L. Carrell or were part of the BNL Crystallographic program library.

References

Cromer, D. T., and Liberman, D. (1970), *J. Chem. Phys. 53*, 915.
Germain, G., Main, P., and Woolfson, M. M. (1971), *Acta Crystallogr. A 27*, 368.
Glusker, J. P., and Marsh, R. M. (1961), *Acta Crystallogr. 14*, 1110.
Huang, P. C. (1971), *Progr. Biophys. Mol. Biol. 23*, 105.
Ivanovics, G. A., Rousseau, R. J., and Robins, R. K. (1971), *J. Med. Chem. 14*, 1155.
Pauling, L., and Corey, R. B. (1951), *Proc. Nat. Acad. Sci. U. S. 37*, 729.
Richards, F. M., Wyckoff, H. W., and Allewell, N. (1970), The Neurosciences, New York, N. Y., Rockefeller University Press, p 901.
Roberts, M., and Visser, D. W. (1952), *J. Biol. Chem. 194*, 695.
Sobell, H. M., Jain, S. C., Sakore, T. D., and Nordman, C. E. (1971), *Nature (London) 231*, 200.
Stewart, R. F., Davidson, E. R., and Simpson, W. T. (1965), *J. Chem. Phys. 42*, 3175.
Sundaralingam, M. (1969), *Biopolymers 7*, 821.
Sundaralingam, M., and Arora, S. K. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1021.
Visser, D. W. (1955), *Antimetabol. Cancer Symp. 1953*, 59.
Visser, D. W., Langenborg, D. L., and Pearson, H. E. (1952), *Proc. Soc. Exp. Biol. Med. 79*, 571.
Voet, D., and Rich, A. (1970), *Progr. Nucl. Acid Res. Mol. Biol. 10*, 183.

A Collagenolytic Protease from the Hepatopancreas of the Fiddler Crab, *Uca pugilator*. Purification and Properties†

Arthur Z. Eisen,* Kenneth O. Henderson, John J. Jeffrey, and Ralph A. Bradshaw‡

ABSTRACT: A collagenolytic protease has been isolated from extracts of the hepatopancreas of the fiddler crab, *Uca pugilator*, and purified to homogeneity by a variety of chromatographic procedures. The enzyme acts both on native collagen fibrils and on collagen in solution and is capable of degrading the collagen molecule under conditions that do not denature the protein. Unlike the vertebrate collagenases the purified crab hepatopancreas collagenase also demonstrates specificities against synthetic substrates for mammalian trypsin and chymotrypsin. These latter enzymes, however, are incapable of cleaving the native collagen helix. In certain respects this collagenase resembles the vertebrate trypsins in that it is inhibited by diisopropyl fluorophosphate, tosyllysyl chloromethyl ketone, and soybean trypsin inhibitor, has the same pH optimum, an approximate minimum molecular weight of 25,000, and a similar amino acid composition. It differs in that it is inactivated at acid pH, is not stabilized by calcium, is an acidic protein, has chymotryptic activity, and has aspartic acid as its apparent amino-terminal group. The findings presented indicate that this unusual enzyme possesses not only specific collagenase activity but also trypsin- and chymotrypsin-like activities as an inherent part of the same molecule.

Although a number of digestive proteases have been demonstrated in a variety of lower animals (Prahl and Neurath, 1966; Gibson and Dixon, 1969; Zwilling *et al.*, 1969; Winter and Neurath, 1970; Camacho *et al.*, 1970), little attention has been given previously to the presence of collagenases in these species even though many of these animals, such as the crustaceans, are predaceous scavengers that feed on animal tissues frequently containing collagen. However, a collagenolytic enzyme has been obtained from the hepatopancreas of the fiddler crab, *Uca pugilator* (Eisen and Jeffrey, 1969), which, unlike most vertebrate collagenases, is readily extractable in its active form (Eisen *et al.*, 1970a).

In common with other collagenases, this enzyme acts on native collagen in solution and is characterized by the cleavage of the native collagen helix at loci 75, 70, and 67% from the amino terminal of the molecule. In addition, there is a marked decrease in the original intramolecular cross-linked β component of collagen with a corresponding increase in the monomeric α chains (Eisen and Jeffrey, 1969).

Early attempts to purify the hepatopancreas collagenase were complicated by the persistence of enzymatic activities possessing specificities normally associated with trypsin and chymotrypsin. The existence of such activities is not unexpected in view of the digestive function of the crab hepatopancreas. Nonetheless, it has been shown that neither vertebrate trypsin nor chymotrypsin, in quantities equal to or greater than that present in hepatopancreas extracts, is capable of cleaving the native collagen helix in either soluble or fibrillar form (Eisen and Jeffrey, 1969).

The first indication that the trypsin and chymotrypsin activities of the collagenase preparations might be inherent to the same enzyme was revealed by inhibitor studies using

† From the Division of Dermatology, Department of Medicine, and the Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110. *Received November 29, 1972.* This investigation was supported by U. S. Public Health Service Grants AM 12129, AM 05611, and AM 13352.

‡ R. A. B. is a recipient of Research Career Development Award AM 23968.

iPr_2FP.[1] Not only did this reagent inactivate the collagenolytic activity, in contrast to its effect on other animal collagenases, but it also abolished the chymotrypsin and trypsin activities. On the other hand, EDTA, which inhibits other animal and human collagenases (Eisen *et al.*, 1970a), was ineffective against all three activities. These observations suggest that the crab hepatopancreas collagenase is a serine protease, probably related either to the vertebrate or bacterial serine proteases (Neurath *et al.*, 1967). In the present study the enzyme has been purified to homogeneity and shown to have multiple specificities which include synthetic substrates containing tryptic and chymotryptic sensitive bonds as well as collagenase activity.

Experimental Procedure

Materials. The fiddler crabs used in this study were obtained from Gulf Specimens Co., Panacea, Fla. Immediately upon arrival the hepatopancreas was dissected from approximately 250 animals and frozen at −70°. In all cases the tissue was processed within 24 hr after removal from the organism.

Tos-Arg-OMe, BzTyrOEt, and AcTyrOEt were obtained from Mann Chemical Co. Soybean trypsin inhibitor (three times crystallized), trypsin (two times crystallized), α-chymotrypsin (three times crystallized), pepsin, ovalbumin, and CHOM were purchased from Worthington Biochemical Corp. and bovine serum albumin was purchased from Sigma Chemical Corp. iPr_2FP (Sigma Chemical Corp.) was diluted to 1 M with anhydrous 2-propanol. 5-Dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) was the product of Sigma Chemical Corp. Standard Dns amino acids were obtained from Schwarz-Mann and polyamide plates from Gallard-Schlesinger Co. Tos-LysCH_2Cl and Tos-PheCH_2Cl were obtained from Calbiochem. *p*-Mercuribenzoate (PMB) was the product of Mann Chemical Corp. All other reagents were of analytical grade and came from a variety of commercial sources.

ε-Aminocaproyl-D-tryptophan methyl ester was graciously supplied by Dr. Pedro Cuatrecasas from the Departments of Medicine and Pharmacology, Johns Hopkins Medical School, Baltimore, Md.

Methods. PREPARATION OF ACETONE POWDER. All procedures in the preparation of the acetone powder were performed at −20°. Frozen tissue was homogenized in approximately 10 vol of acetone in a Waring Blendor for 5 min. The material was centrifuged at 12,000*g*, the acetone was removed by suction, and the pellets were suspended in another 10 vol of butanol followed by a similar quantity of acetone. The preparations were dried in a vacuum desiccator over H_2SO_4 for 24 hr and then stored *in vacuo* at 4° in the presence of anhydrous $CaSO_4$.

ASSAY PROCEDURES. Collagenase activity was determined by a method that depends on the release of soluble [^{14}C]glycine-containing peptides from native, reconstituted, guinea pig skin collagen fibrils (Nagai *et al.*, 1966). Guinea pig skin collagen was purified by the method of Gross (1958) and reconstituted native fibrils were prepared from solutions of radioactive collagen to accommodate smaller volumes of radioactive collagen and enzyme solutions as described previously (Tokoro *et al.*, 1972). A unit of enzyme activity was defined as the release of 0.01 μg of collagen/min.

Collagenase activity was also assessed by measurements of specific viscosity as a function of time. Viscosity measurements were made in Ostwald viscometers with flow rates at 25° ranging from 26 to 32 sec using enzyme–collagen reaction mixtures as previously described (Eisen and Jeffrey, 1969) for the crude crustacean collagenase.

BzTyrOEt (or less frequently AcTyrOEt) was employed to assay for chymotrypsin-like activity (Schwert and Takenaka, 1955) by measuring the rate of hydrolysis of 0.001 M BzTyrOEt in 0.05 M Tris-HCl, pH 8.0, at 237 nm, 25°. Trypsin-like activity was determined by measuring the rate of hydrolysis of Tos-ArgOMe, 0.001 M in 0.05 M Tris-HCl, pH 8.0, containing 0.01 M $CaCl_2$ at 247 nm, 25° (Hummel, 1959). In both cases a unit of enzyme activity is defined as a change in absorbance of 0.001 unit/min at 25°.

Protein was determined by the method of Lowry *et al.* (1951) or by the procedure of Warburg and Christian (1941) which is based on the absorbance at 280 and 260 nm.

CHROMATOGRAPHY. All chromatographic procedures were carried out at 4°. Sephadex G-150 (Pharmacia) was equilibrated in 0.05 M Tris-HCl, pH 7.5, containing 0.2 M sodium chloride. Sephadex G-75 (Pharmacia) was equilibrated in 0.05 M Tris-HCl, pH 7.5. Ion exchange chromatography was performed on DE-32 microgranular cellulose (Whatman) equilibrated in 0.005 M Tris-HCl, pH 7.5, containing 0.15 M sodium chloride. Hydroxylapatite (Bio-Rad) was washed in 0.5 M phosphate buffer, pH 6.9, and equilibrated in 0.005 M phosphate buffer, pH 6.9. Sepharose 4B (Pharmacia) was washed thoroughly with distilled water and activated with cyanogen bromide (Eastman) as described by Cuatrecasas *et al.* (1968). Approximately 100 mg of ε-aminocaproyl-D-tryptophan methyl ester was coupled to 10 ml of packed Sepharose.

ELECTROPHORESIS. For monitoring enzyme purification, disc gel electrophoresis was carried out according to the method of Davis (1964) using a 10% concentration of acrylamide. Thermally denatured collagen in reaction mixtures was subjected to electrophoresis in polyacrylamide gels according to Nagai *et al.* (1964). The enzyme was inactivated by the addition of sufficient 0.1 M HCl to the reaction mixture to reduce the pH to approximately 2 (0.03 M) before denaturation at 40°.

Gel electrophoresis in sodium dodecyl sulfate was performed by a modification of the method of Dunker and Rueckert (1969) in Tris-glycine buffer, pH 8.5, using 15% acrylamide gels. Electrophoresis was carried out for 8 hr at room temperature at a current of approximately 3 mA/tube. Proteins were stained with 0.25% Coomassie Blue (Fairbanks *et al.*, 1971).

ULTRACENTRIFUGATION. Ultracentrifugation studies were performed in a Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanning system. Sedimentation velocity runs were performed in a double sector cell at 59,000 rpm at 4–6°. Sedimentation coefficients were determined by means of the schlieren patterns on photographic plates which were analyzed by the use of a Nikon microcomparator.

Sedimentation equilibrium experiments were carried out at 6° utilizing an ultraviolet scanner at 280 nm and a speed of 36,000 rpm by the method of Yphantis (1964). Attainment of equilibrium was determined when no further change in pattern was observed. The data were traced into a digital computer and the molecular weight was computed by the best least-squares fit of the slope of log optical density *vs.* distance squared (x^2) from the center of the rotor.

[1] Abbreviations used are: BzTyrOEt, *N*-benzoyl-L-tyrosine ethyl ester; AcTyOEt, *N*-acetyl-L-tyrosine ethyl ester; Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; CHOM, chicken ovomucoid; Tos-LysCH_2Cl, tosyllysyl chloromethyl ketone; Tos-PheCH_2Cl, tosylphenylethyl chloromethyl ketone; PMB, *p*-mercuribenzoate; iPr_2FP, diisopropyl fluorophosphate.

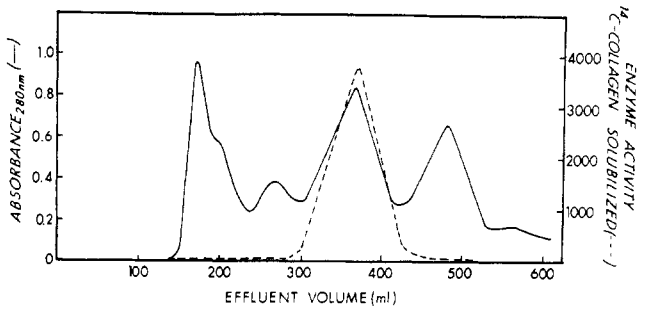

FIGURE 1: Gel filtration of an ammonium sulfate preparation of hepatopancreas collagenase containing approximately 250 mg of enzyme protein in a volume of 7 ml on Sephadex G-150 using reverse flow. A column (2.5 × 100 cm) was equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.005 M $CaCl_2$. Effluent fractions of 5 ml were collected at a rate of 15 ml/hr.

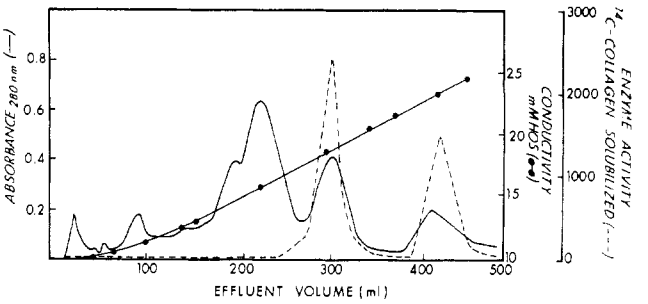

FIGURE 2: DEAE-cellulose chromatography of the active fraction from gel filtration. Approximately 95 mg of enzyme protein in 10 ml of 0.005 M Tris-HCl, pH 7.5, containing 0.15 M NaCl was pumped onto a column (1.2 cm × 25 cm) at a rate of 25 ml/hr and effluent fractions of 5 ml were collected. The enzyme was eluted using a linear NaCl gradient from 0.15 to 0.65 M. Total gradient volume was 500 ml.

AMINO ACID ANALYSIS. Analyses were performed by the method of Spackman *et al.* (1958) with the Beckman Model 121 fully automated amino acid analyzer. Samples were hydrolyzed *in vacuo* with 6 N HCl at 110° for 24, 48, or 72 hr. Tryptophan was determined by the method of Edehoch (1967). Half-cystine was determined as cysteic acid as described by Moore (1963).

AMINO-TERMINAL ANALYSES. Qualitative amino-terminal analyses of the purified collagenolytic protease were carried out by the dansylation method as described by Weiner *et al.* (1972). Ascending chromatography on polyamide plates was performed using the solvent systems reported. Quantitative amino-terminal analyses of the purified enzyme were determined on protein denatured in urea by the method of Stark and Smyth (1963) and in sodium dodecyl sulfate as modified by Pétra and Neurath (1969).

IMMUNOLOGIC STUDIES. Adult male white rabbits weighing 5–6 kg were injected initially with 0.8 mg of the purified collagenolytic protease in complete Freund's adjuvant. Rabbits whose sera showed precipitating antibodies were given a booster injection at 3 weeks and bled again 1 week later. Sera were pooled and taken to 33% saturation at 0° with $(NH_4)_2SO_4$, pH 7.0, to precipitate the γ-globulin fraction (Bauer *et al.*, 1970). The $(NH_4)_2SO_4$ precipitate was dissolved in 0.05 M Tris-HCl, pH 7.5, containing 0.1 M NaCl, dialyzed against the same buffer, and adjusted to a concentration of 25 mg of protein/ml before use. Gel diffusion was performed according to the method of Ouchterlony, 1958.

OPTICAL ROTATION. Optical rotation was measured, in parallel with viscosity measurements, in a Cary 60 automatic spectropolarimeter at 230 nm at temperatures ranging from 25 to 28°.

Results

Purification of the Collagenolytic Protease. Approximately 3 g of acetone powder was suspended in 35 ml of cold 0.05 M Tris-HCl, pH 7.5, containing 0.005 M $CaCl_2$ by stirring for 1 hr. The suspension was centrifuged at 39,100*g* for 20 min and the residue reextracted with an equal volume of the same buffer for 30 min and centrifuged, and the two supernatants were combined. The enzyme prepared in this fashion appeared to be present in a fully active form rather than as a zymogen. Following the addition of trypsin to these extracts at final concentrations of 0.05–0.2 mg/ml, the samples were subsequently incubated at either 4 or 25° for varying times. No increase in activity was observed when the reaction mixtures were then examined for collagenolytic-, tryptic-, or chymotryptic-like activities. No further studies were done to determine whether or not the enzyme is synthesized in a zymogen form.

The enzyme extract was brought to a final saturation of 70% by the addition of a solution of saturated ammonium sulfate adjusted to pH 7.5 with concentrated NaOH at 0°. The precipitate was collected by centrifugation at 39,100*g* for 30 min and dissolved in 20 ml of 0.05 M Tris-HCl, pH 7.5, containing 0.005 M $CaCl_2$.

Gel filtration of the redissolved precipitate was performed by reversed flow on a column (2.5 × 100 cm) of Sephadex G-150 equilibrated with the initial Tris–calcium buffer. As shown in Figure 1, a single peak of collagenolytic activity was obtained. The collagenolytic activity elutes exactly with the tryptic- and chymotryptic-like activities which are not depicted in Figure 1. The active fractions were pooled, dialyzed against distilled water, and lyophilized. Extreme care was required during dialysis so that the enzyme solution was not exposed to dialysis tubing for periods longer than 6 hr. If this precaution was not followed, the dialysis tubing became extremely friable and the enzyme solution could be lost from the bag. This observation suggests that the enzyme fraction at this stage of purity contains a cellulase capable of degrading the dialysis tubing.

Ion exchange chromatography was carried out on a column of microgranular DEAE-cellulose (Whatman DE-32) equilibrated in 0.005 M Tris-HCl, pH 7.5, containing 0.15 M NaCl (starting buffer). The lyophilized enzyme powder obtained from gel filtration was dissolved in the starting buffer, dialyzed against the same buffer for 3 hr, and pumped onto a DE-32 column (1.2 cm × 30 cm) at a rate of 25 ml/hr. The enzyme was eluted using a linear gradient established from the starting buffer to 0.65 M NaCl. Chromatography on DEAE-cellulose (Figure 2) results in the separation of the collagenolytic activity into two components referred to as peak 1 and peak 2. Both peaks 1 and 2 contain not only collagenolytic activity but also retain trypsin- and chymotrypsin-like activities. Since peak 2 has significantly less protein than peak 1 and was found to be considerably enriched in trypsin-like activity relative to its collagenolytic activity, only peak 1 was examined in detail. However, it should be noted (*vide infra*) that peak 2 is closely related to peak 1. The differences characterizing these two forms of the enzyme have not been determined.

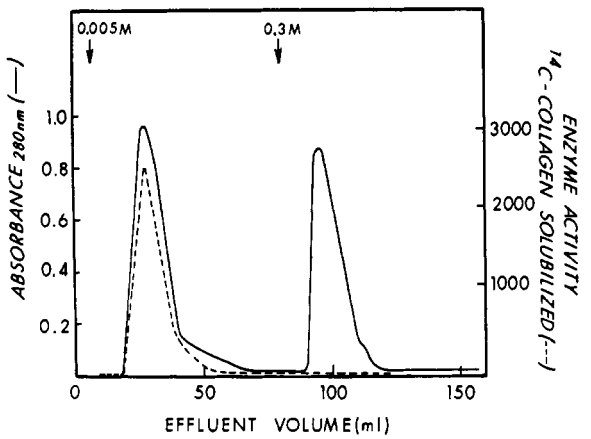

FIGURE 3: Hydroxylapatite chromatography of peak 1 from DEAE-cellulose. The column, 1.5 × 8 cm, was equilibrated with 0.005 M phosphate buffer, pH 6.9, and the enzyme sample applied in 5 ml of the same buffer. The flow rate was 33 ml/hr and the fraction size was 3.5 ml.

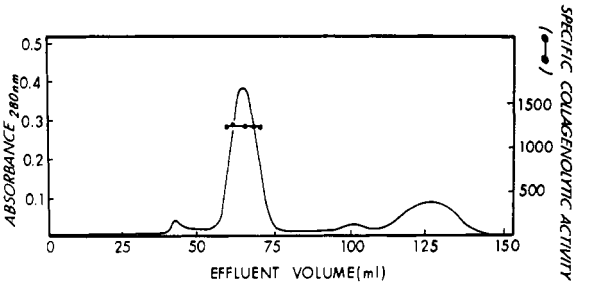

FIGURE 4: Gel filtration on Sephadex G-75 of the partially purified hepatopancreas collagenase. A column (1.5 × 90 cm) was equilibrated with 0.05 M Tris-HCl, pH 7.5, and the sample containing 11 mg of protein was applied in a volume of 3 ml. Effluent fractions of 4 ml were collected at a rate of 24 ml/hr.

Fractions from peak 1 containing collagenase activity were pooled, dialyzed against distilled water, and lyophilized. At this stage of purification the cellulase activity was lost and dialysis could be carried out without damage to the dialysis tubing.

The concentrated enzyme powder was dissolved in 0.005 M phosphate buffer, pH 6.9, and applied to a column of hydroxylapatite (1.5 × 8 cm) equilibrated in the same buffer. A larger column (2.5 × 14 cm) was used for preparative purposes when pooled material from several DEAE columns was used. Figure 3 shows the results of a typical separation. The active enzyme fraction, containing collagenolytic-, tryptic- and chymotryptic-like activities, emerged as a single peak with the void volume. The combined eluates (from 20 to 40 ml) were pooled, dialyzed, and lyophilized.

In an effort to isolate the chymotrypsin from the collagenase and trypsin, the enzymatically active peak obtained from hydroxylapatite was subjected to affinity chromatography using Sepharose 4B coupled with ε-aminocaproyl-D-tryptophan methyl ester as described for the purification of bovine chymotrypsin by Cuatrecasas *et al.* (1968). The Sepharose column (1.2 × 5 cm) was equilibrated with 0.05 M Tris-HCl, pH 8.0. Approximately 10–15 mg of partially purified enzyme was applied in 1.0 ml of the same buffer. Elution with 0.1 M acetic acid, pH 3.0, was unsuccessful but a single protein peak was obtained when a buffered solution of 1 M NaCl was used as the eluent. This peak contained not only collagenase activity but also retained the chymotrypsin- and trypsin-like activities. Examination of the enzymatically active fraction on disc gel electrophoresis revealed a single protein band migrating above the buffer front. Although affinity chromatography resulted in a highly purified enzyme preparation, only partial adsorption of the total enzyme activity applied to the column (40%) occurred and yields of the purified protein were low.

To improve the yield of purified enzyme, the lyophilized product of the hydroxylapatite column was subjected to gel filtration on a column (1.5 × 90 cm) of G-75 equilibrated with 0.05 M Tris-HCl, pH 7.5. As shown in Figure 4 the enzyme was eluted in a single symmetrical peak (eluent fractions 15–21) with constant collagenolytic specific activity in each fraction. Both trypsin- and chymotrypsin-like activities were also constant in each fraction across the peak. The yields and purification of a typical enzyme preparation are summarized in Table I.

Examination of the enzymatically active fraction from gel filtration on G-75 by disc electrophoresis in 10% polyacrylamide at pH 8.3 demonstrated a single sharp protein band (Figure 5). In addition, no evidence of impurity was noted when disc gel electrophoresis was carried out at pH 4.3 or when gel electrophoresis was performed in sodium dodecyl sulfate.

This electrophoretically pure protein was used for immunization and as shown in Figure 6A the antihepatopancreas collagenase antibody gave a single precipitin band when allowed to react with its purified antigen or with a crude enzyme solution, thus providing additional evidence for the purity of the enzyme. It is of interest that when the purified enzyme and the material present in peak 2 from DEAE-cellulose chromatography were allowed to react with the antihepatopancreas collagenase antibody, a reaction of complete identity occurred (Figure 6B) indicating that the two enzymes share major antigenic determinants. No reactions were seen on immunodiffusion when the antihepatopancreas collagenase antibody was allowed to react with human, animal, or bacterial collagenases or with bovine trypsin and chymotrypsin. Additional studies, such as those performed by Arnon and Neurath (1969), will be required to demonstrate possible cross-reactivity between the crustacean collagenase and other serine proteases.

TABLE 1: Purification of Fiddler Crab Hepatopancreas Collagenase.

Purification Step	Total Protein (mg)	Sp Act.[a]	Total Act. (×10³)	Yield (%)
Crude	2250	125	281	100
$(NH_4)_2SO_4$ ppt	1138	226	257	92
Sephadex G-150	270	558	159	57
DEAE-cellulose				
peak 1	48	2937	141	50
peak 2	12	734		
Hydroxylapatite	11	3875	43	15
Sephadex G-75	5	4020	20	7

[a] Specific activity refers to the micrograms of collagen solubilized per minute per milligram of protein.

FIGURE 5: Polyacrylamide gel electrophoresis of purified hepatopancreas collagenase. Approximately 80 μg of protein was applied to the upper gel. Electrophoresis was carried out in 10% acrylamide at a constant current of 5 mA/tube.

Analytical Ultracentrifugation Studies. At pH 7.6 in 0.05 M Tris-HCl buffer the purified enzyme preparation sedimented as a single symmetrical peak with an $s_{20,w}$ of 2.3 at a protein concentration of 2.0 mg/ml. Sedimentation equilibrium experiments were carried out at pH 7.6 using initial protein concentrations of 0.25, 0.4, and 0.6 mg/ml. A plot of log C *vs.* x^2 was linear providing further evidence for the homogeneity of the enzyme preparation. The apparent molecular weight calculated from the slope of this line using a value of 0.726 for the partial specific volume, determined from the amino acid composition (*vide infra*), gave molecular weight values of 26,890 (at 0.25 mg of protein/ml), 25,010 (at 0.4 mg of protein/ml), and 24,700 (at 0.6 mg of protein/ml).

The molecular weights obtained by ultracentrifugation were in close agreement with the approximate molecular weights determined by means of gel filtration on Sephadex G-150 (Andrews, 1964) and by gel electrophoresis in sodium dodecyl sulfate. The values obtained were 25,00 and 25,700, respectively.

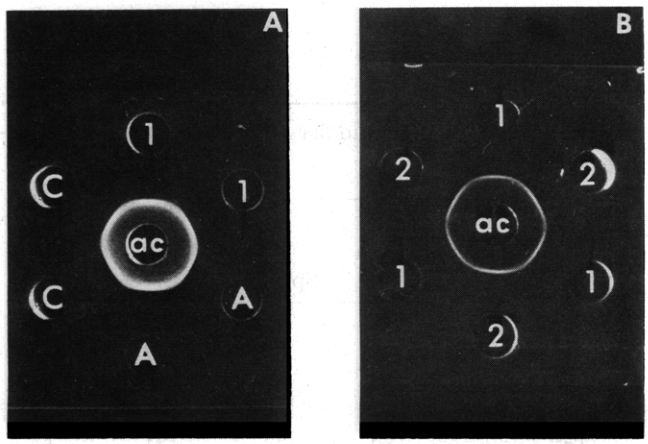

FIGURE 6: Gel diffusion analysis of hepatopancreas collagenase against antihepatopancreas collagenase antibody. (A) Antiserum to *Uca pugilator* hepatopancreas collagenase, ac, in center well: 1, electrophoretically pure hepatopancreas collagenase; C, crude enzyme from an acetone powder extract; A, ammonium sulfate fraction of hepatopancreas collagenase. (B) ac, antihepatopancreas collagenase antibody: 1, peak 1 from DEAE-cellulose chromatography; 2, peak 2 from DEAE-cellulose chromatography.

TABLE II: Amino Acid Composition of Fiddler Crab Hepatopancreas Collagenase.[a]

Amino Acid	Time of Hydrolysis (hr) 24[b]	48[b]	72[b]	Av or Extrapoln	Integral No.
Lysine	1.7	1.8	1.5	1.6	2
Histidine	4.8	4.8	4.8	4.8	5
Arginine	4.4	4.5	4.6	4.5	5
Aspartic acid	29.2	28.5	29.2	28.9	29
Threonine[c]	22.7	21.7	21.1	23.7	24
Serine[c]	15.3	13.6	13.2	16.7	17
Glutamic acid	12.9	13.1	12.9	13.0	13
Proline	12.0	12.5	12.7	12.4	12
Glycine	27.6	28.6	28.5	28.2	28
Alanine	21.0	21.0	21.0	21.0	21
Half-cystine[d]				8.0	8
Valine	19.7	20.1	20.4	20.4	20
Methionine	2.7	2.9	2.8	2.8	3
Isoleucine	16.0	16.4	16.6	16.6	17
Leucine	13.1	13.6	13.6	13.4	13
Tyrosine[c]	7.7	7.6	7.3	8.0	8
Phenylalanine	8.2	8.2	8.4	8.3	8
Tryptophan[e]				2.3	2
Total					235

[a] Residues are given in residues/molecule assuming a mol wt of 25,500. [b] Average of three determinations. [c] Extrapolated to zero time of hydrolysis. [d] Determined as cysteic acid by the method of Moore (1963). [e] Determined spectrophotometrically by the method of Edehoch (1967).

Amino Acid Composition. Amino acid analyses were performed as described under Methods and the results are shown in Table II. Values are presented as residues/molecule assuming a mol wt of 25,500. Titration with PMB (Boyer, 1954) showed no free sulfhydryl groups suggesting that all of the half-cystine is present in disulfide linkage.

Effect of Inhibitors. The ability of various classes of inhibitors and inactivators to block the action of the hepatopancreas collagenolytic protease on native collagen fibrils is shown in Table III. iPr_2FP, Tos-LysCH_2Cl, and soybean trypsin inhibitor produce almost complete inhibition of the collagenolytic activity. Surprisingly, Tos-PheCH_2Cl was virtually without effect. EDTA and cysteine have no effect on the hepatopancreas enzyme which is in contrast to the ability of these agents to inhibit bacterial as well as most animal and human collagenases (Eisen *et al.*, 1970a; Tokoro *et al.*, 1972). Human serum, a known inhibitor of vertebrate collagenases (Eisen *et al.*, 1970a,b), also inhibits the hepatopancreas collagenase but only at approximately 100-fold greater serum protein to enzyme levels.

The action of these reagents on the tryptic- and chymotryptic-like activities was entirely parallel. Soybean trypsin inhibitor completely inhibited both activities within 10 min at a molar ratio of inhibitor to enzyme of approximately 1.0. Under similar conditions chicken ovomucoid inhibited 85% of the enzyme activity. iPr_2FP, at a molar ratio of 10:1, and Tos-LysCH_2Cl, at a molar ratio of 100:1, completely inactivated

TABLE III: Effect of Inhibitors on Fiddler Crab Collagenase Activity.[a]

Inhibitor	Concn	cpm Solubilized (Above Blank)	% Inhibition
Enzyme control		2927	
iPr_2FP	10^{-3} M	82	97.2
Tos-LysCH_2Cl	10^{-3} M	228	92.2
Tos-PheCH_2Cl	10^{-3} M	2922	0
Soybean trypsin inhibitor	0.1 mg/ml	58	98.0
Cysteine	10^{-2} M	2710	7.4
EDTA	5×10^{-3} M	2934	0
Serum[b]	0.4 mg/ml	2567	12.3
Serum	4.0 mg/ml	1413	55.1
Serum	40.0 mg/ml	0	100

[a] Reaction mixtures contained 50 μl of 0.2% [^{14}C]glycine-labeled collagen as a substrate gel (3820 cpm/gel) and 25 μg of enzyme protein in a total volume of 200 μl. Incubation was carried out at 37° for 90 min in a shaken water bath. 0.01% trypsin blanks represent approximately 8% of the counts in the substrate gel. [b] Serum concentrations represent a 1:1000, 1:100, and a 1:10 dilution, respectively, of whole serum.

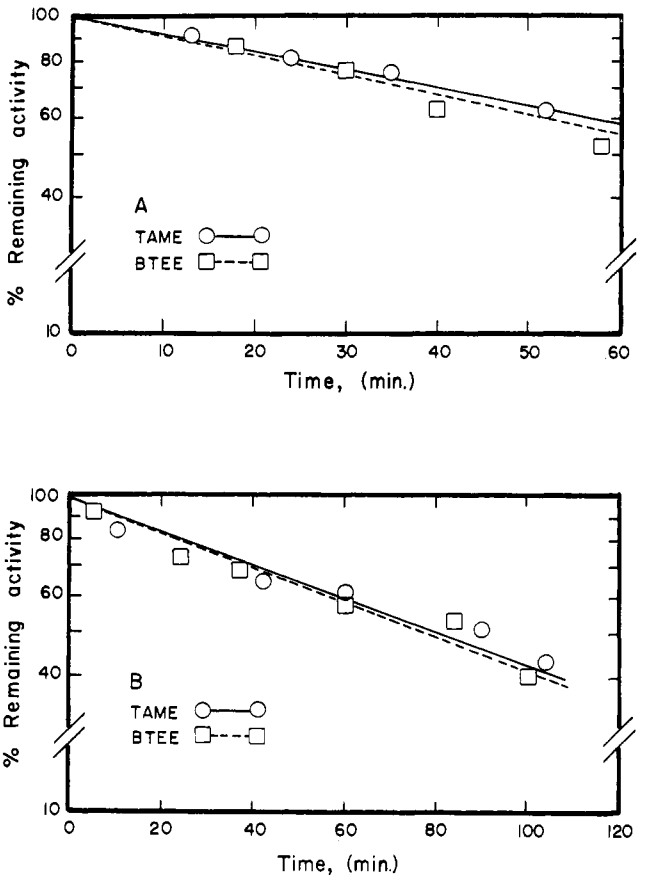

FIGURE 7: Kinetics of the inhibition of the hydrolysis of Tos-Arg-OMe and BzTyrOEt by crab hepatopancreas collagenase using (A) iPr_2FP, 10^{-5} M, in the presence of 3×10^{-5} M enzyme; (B) Tos-Lys-CH_2Cl, 5×10^{-3} M in the presence of 2×10^{-5} M enzyme; temperature 25°; pH 8.0. Values presented as the log of the per cent remaining activity.

both of these enzymes in 20 min, pH 8.0. The kinetics of the reactions with iPr_2FP (Figure 7A) and Tos-LysCH_2Cl (Figure 7B) show simultaneous inhibition of the trypsin- and chymotrypsin-like activities of the enzyme yielding curves that are virtually superimposable.

As with the collagenolytic activity, Tos-PheCH_2Cl, a potent inhibitor of chymotrypsin, had no inhibitory effect on any activity of the enzyme even at a molar ratio of 50:1.

In addition, neither cysteine nor EDTA had an inhibitory effect on the trypsin- or chymotrypsin-like activities. In contrast to bovine trypsin, calcium had no effect on the stability of the enzyme at pH 8.0, 25°, and at concentrations ranging from 0.01 to 0.2 M for incubation times up to 48 hr.

pH Optima. The optimum for the collagenase activity was measured by the release of soluble radioactivity from ^{14}C-labeled reconstituted collagen fibrils and for tryptic- and chymotryptic-like activities using 10^{-2} M Tos-Arg-OMe and BzTyrOEt, respectively. The buffers used to obtain the appropriate pH values in the range of 5–9 were citrate–phosphate and Tris-HCl at a final concentration of 0.05 M. The final pH at each point was determined using appropriate blanks in the absence of enzyme protein. Figure 8 shows that the pH optima for the three enzyme activities is approximately 8.0 with almost complete loss of esterase activity below pH 6.0. A low but significant level of collagenase activity is still present at pH 6 but is virtually abolished below pH 5.0. Below pH 3.5 there is an irreversible loss of enzyme activity.

Optical Rotation and Disc Electrophoresis of Enzyme–Collagen Reaction Mixtures. The purified enzyme is capable of reducing the initial specific viscosity of a collagen at 25° by approximately 50% in 3 hr and by greater than 80% at the end of 8 hr of incubation. No accompanying change in optical rotation was noted during this period of observation. These results are identical with those reported in detail previously for the crude enzyme (Eisen and Jeffrey, 1969). At 28° the rate of enzyme activity was much greater than at 25° with a loss of more than 75% of the initial specific viscosity in 3 hr. Measurements of optical rotation during this time period at 28° indicate a loss of approximately 2%/hr of the helical content. A similar effect of temperature on optical rotation was reported for rat skin collagenase (Tokoro *et al.*, 1972) and has been attributed to the instability of the small new fragments formed as TC^A_{75} undergoes further cleavage.

When the denatured products of enzymatic attack by the pure collagenolytic protease on the collagen molecule were examined by acrylamide gel electrophoresis from reaction mixtures incubated at 25°, the number and relative size of the collagen fragments formed were virtually identical with those reported for the crude enzyme (Eisen and Jeffrey, 1969). At a loss of approximately 20% in specific viscosity there was a marked increase in the original β band, a corresponding increase in the density of the α band, and the appearance of new bands beneath the original β and α components. Figure 9 illustrates the electrophoretic pattern of a pure enzyme–collagen reaction mixture, which has lost 70% of its initial specific viscosity and demonstrates clearly the marked decrease in the original β band and the numerous new components beneath the original α band. The TC^A_{25} bands are also visible above the buffer front. Identical patterns were obtained at 28°.

Amino-Terminal Analysis. Qualitative determination of the amino-terminal residue of the hepatopancreas collagenolytic

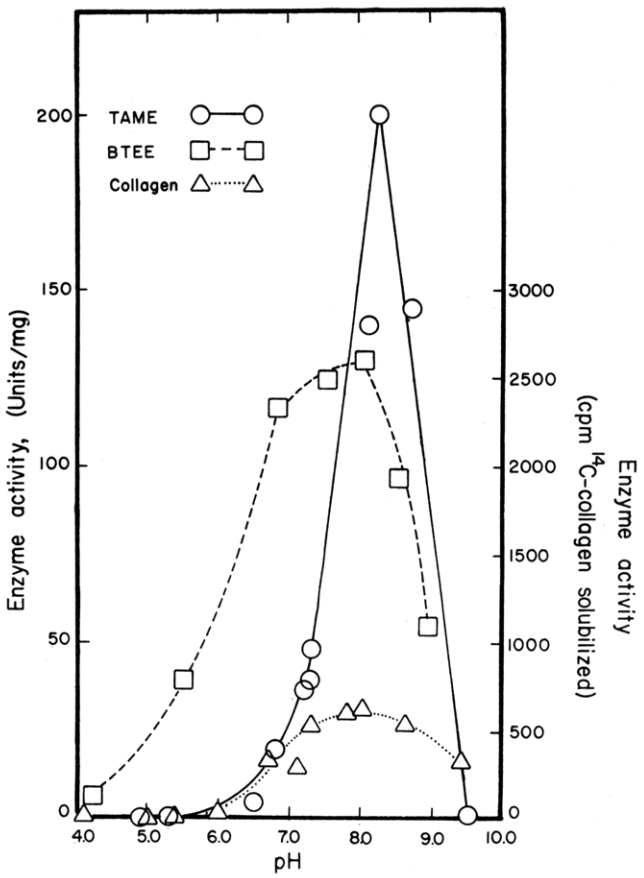

FIGURE 8: Effect of pH on enzyme activity measured by the release of soluble radioactivity from ^{14}C-labeled reconstituted collagen fibrils and the hydrolysis of Tos-Arg-OMe and BzTyrOEt. See text for assay conditions.

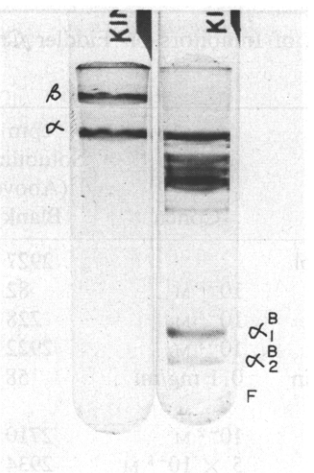

FIGURE 9: Acrylamide gel electrophoretic pattern of thermally denatured enzyme–guinea pig skin collagen reaction mixtures at 25°. On the left, zero time reaction mixture, and, on the right, after 70% reduction in specific viscosity. α refers to the single polypeptide chains β to the cross-linked dimers of the α chains; α_1^B and α_2^B refer to the one-quarter length fragments (TC^{B}_{25}); F refers to the buffer front.

protease was carried out by dansylation followed by chromatography on polyamide plates. This procedure revealed only aspartic acid at the amino terminus of the molecule. On no occasion were multiple spots observed from several different enzyme preparations examined. Determination of the amino-terminal residue of proteins whose sequence is known provided the expected result in all cases. These findings support the previous conclusion that the enzyme is a homogeneous preparation.

Quantitative amino-terminal analyses by the method of Stark and Smyth (1963), however, yielded not only 0.3 mol of aspartic acid but also 0.4 mol of glycine per mole of protein. Smaller amounts of serine and glutamic acid, approximately 0.15 mol of protein, were also detected. Due to the difficulty in removing all of the pyrrolidinecarboxylic acid arising from internal glutamine and the high correction factor for serine (×5), the significance of these values is marginal.

These results suggest that limited autolysis of the collagenolytic protease, probably at one site, can occur either as a result of the preparative procedure used or during the carbamylation reaction of the end group procedure. The latter may occur even though the enzyme was pretreated with iPr_2FP prior to the analysis, since trace amounts of native enzyme would be sufficient to generate the observed heterogeneity. The net effect of internal proteolysis, no matter where it occurs, would be to largely destroy the quantitation because of the variable loss of fragments during dialysis of the carbamyl protein. Although this hypothesis is favored because of the single band seen on disc gels of the native enzyme, the extent, if any, to which the hepatopancreas collagenase contains internal cleavages, such as have been noted for trypsin (Smith and Shaw, 1969), is being investigated.

Discussion

The collagenolytic protease from the hepatopancreas of the crustacean *Uca pugilator* has now been purified to homogeneity by electrophoretic, ultracentrifugation, and immunologic criteria. The purified enzyme is unique in that it not only behaves as a specific collagenase but also possesses trypsin- and chymotrypsin-like activities as an inherent part of the molecule. Although the crustacean hepatopancreas enzyme has esterolytic activity characteristic of chymotrypsin against synthetic substrates, this capability is significantly less (by approximately (five- to sevenfold) than its ability to hydrolyze trypsin substrates. Of interest is the observation that Tos-PheCH_2Cl, a potent inhibitor of mammalian and certain invertebrate chymotrypsins, fails to inhibit the hepatopancreas protease. The reason for this remains obscure. It should also be noted that the tryptic and chymotryptic specificities of the hepatopancreas enzyme have only been defined for synthetic, single amino acid ester substrates and that ultimately protein substrates must be used to determine how precisely the cleavage specificities of this enzyme parallel those of vertebrate trypsin and chymotrypsin.

A number of trypsin-like enzymes from the digestive organs of certain invertebrates have been characterized (Winter and Neurath, 1970; Zwilling *et al.*, 1969; Camacho *et al.*, 1970) but they demonstrate no chymotrypsin activity and have not, as yet, been explored for collagenase activity. In addition, a chymotrypsin-like protease has been isolated from the gastric filaments of the coelenterate, *Metridium senile* (Gibson and Dixon, 1969), which appears to have only a single specificity.

In many respects the collagenolytic protease from the crustacean hepatopancreas resembles the mammalian trypsins

in that it is inhibited by iPr_2FP and Tos-LysCH_2Cl, as well as by soybean trypsin inhibitor and chicken ovomucoid, and has the same pH optimum and approximately the same molecular weight. However, the collagenolytic protease also differs from mammalian trypsin in a number of its properties. The enzyme is inactivated at acid pH, is not stabilized by calcium, and, based on its amino acid composition and strong affinity for DEAE-cellulose, is acidic in nature. In these characteristics the enzyme is similar to a number of other invertebrate digestive proteases which have been isolated (Zwilling *et al.*, 1969; Winter and Neurath, 1970; Camacho *et al.*, 1970).

In view of these similarities and differences, it is worthwhile considering whether the hepatopancreas collagenase from *Uca pugilator* is a member of either the family of vertebrate serine proteases, which have been shown by primary structure homology to be related to proteolytic enzymes in lower invertebrates (Neurath *et al.*, 1970) or to the serine protease family represented by the subtilisins which are mechanistically similar but structurally unique (Smith *et al.*, 1970). Alternatively, it may represent yet a third class of enzymes that function *via* active serine residues but is unrelated by obvious evolutionary pathways to either of the other two families.

Despite the differences noted it is most likely that the hepatopancreas collagenase is related to the vertebrate serine proteases. Comparison of the amino acid composition of the enzyme from *Uca pugilator* hepatopancreas to other serine proteases (Table IV) indicates a distinct similarity between the crustacean collagenase and starfish trypsin, an enzyme which has been suggested to bear an evolutionary relationship to bovine trypsin (Winter and Neurath, 1970). The hepatopancreas collagenase is, however, unlike subtilisin in its amino acid composition.

If this enzyme is indeed related to the vertebrate serine proteases, it is interesting to note that the apparent amino-terminal group of the chain, as indicated by dansylation, is aspartic acid. This finding is in contrast to the commonly encountered valine or isoleucine end group of this family which has been shown to be of importance mechanistically as a feature of the activation of the corresponding zymogen. Thus, the presence of amino-terminal aspartic acid would imply that this enzyme does not exist as a zymogen, which is in agreement with direct measurements.

As emphasized previously (Eisen and Jeffrey, 1969) the collagenolytic enzyme from the hepatopancreas of *Uca pugilator* fits the definition of a collagenase in that it is capable of degrading the polypeptide backbone of the collagen molecule under conditions that do not denature the protein. Even though the hepatopancreas collagenase is a digestive rather than a morphogenetic enzyme it cleaves the collagen molecule at the TC^{A}_{75} locus, identically with that of other animal and human collagenases (Eisen *et al.*, 1970a). After the initial scission in the molecule, however, the enzyme continues to digest the cut end of TC^{A}_{75} producing numerous smaller fragments in a similar fashion to that of certain other tissue collagenases (Eisen *et al.*, 1970a; Tokoro *et al.*, 1972). It is important to note that trypsin and chymotrypsin from vertebrate sources are incapable of cleaving the native collagen helix. However, chymotrypsin does attack peptide bonds from the nonhelical region at the amino-terminal end of the collagen molecule at or near the site of the intramolecular cross-link (Bornstein *et al.*, 1966). It is likely then that the conversion of β to α components by this collagenase is related to its chymotrypsin-like activity.

Future sequence studies designed to elucidate the structure of this enzyme will provide an opportunity to inquire further into the mechanism whereby proteolytic enzymes can specifically cleave the collagen molecule and indicate whether the protein is another member of a previously defined family of serine proteases, or a unique species.

TABLE IV: Amino Acid Composition of Fiddler Crab Collagenase to Other Serine Proteases.

	Residues/Molecule				
Amino Acid	Crab Collagenase[a]	Starfish Trypsin[b]	Bovine Trypsin[c]	Chymotrypsin A[d]	Subtilisin BPN[e]
Lysine	2	9	14	14	11
Histidine	5	4	3	2	6
Arginine	5	4	2	3	2
Aspartic acid	29	31	22	22	29
Threonine	24	15	10	22	13
Serine	17	18	33	27	37
Glutamic acid	13	20	14	15	15
Proline	12	13	9	9	14
Glycine	28	28	25	23	33
Alanine	21	16	14	22	37
Half-cystine	8	8	12	10	0
Valine	20	19	17	23	30
Methionine	3	2	2	2	5
Isoleucine	17	11	15	10	13
Leucine	13	13	14	19	15
Tyrosine	8	8	10	4	10
Phenylalanine	8	5	3	6	3
Tryptophan	2	9	4	8	3
Total	235	233	223	241	276

[a] From Table II. [b] Winter and Neurath (1970). [c] Walsh and Neurath (1964). [d] Hartley (1964). [e] Markland and Smith (1967).

References

Andrews, P. (1964), *Biochem. J. 91*, 222.

Arnon, R., and Neurath, H. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1323.

Bauer, E. A., Eisen, A. Z., and Jeffrey, J. J. (1970), *Biochem. Biophys. Acta 206*, 152.

Bornstein, P., Kang, A. H., and Piez, K. A. (1966), *Biochemistry 5*, 3803.

Boyer, P. D. (1954), *J. Amer. Chem. Soc. 76*, 4331.

Camacho, Z., Brown, J. R., and Kitto, G. B. (1970), *J. Biol. Chem. 245*, 3964.

Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 636.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244*, 5074.

Edehoch, J. (1967), *Biochemistry 6*, 1948.

Eisen, A. Z., Bauer, E. A., and Jeffrey, J. J. (1970a), *J. Invest. Dermatol. 55*, 359.

Eisen, A. Z., Bloch, K. J., and Sakai, T. (1970b), *J. Lab. Clin. Med. 75*, 258.

Eisen, A. Z., and Jeffrey, J. J. (1969), *Biochim. Biophys. Acta 191*, 517.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971),

Biochemistry 10, 2606.
Gibson, D. M., and Dixon, G. H. (1969), *Nature (London) 222*, 753.
Gross, J. (1958), *J. Exp. Med. 107*, 247.
Hartley, B. S. (1964), *Nature (London) 201*, 1284.
Hummel, B. C. (1959), *Can. J. Biochem. Physiol. 37*, 1393.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 143*, 265.
Markland, F. S., and Smith, E. L. (1967), *J. Biol. Chem. 242*, 5198.
Moore, S. (1963), *J. Biol. Chem. 138*, 235.
Nagai, Y., Gross, J., and Piez, K. A. (1964), *Ann. N. Y. Acad. Sci. 121*, 494.
Nagai, Y., Lapiere, C. M., and Gross, J. (1966), *Biochemistry 5, 3123*.
Neurath, H., Bradshaw, R. A., and Arnon, R. (1970), *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., New York, N. Y., Academic Press, p 113.
Neurath, H., Walsh, K. A., and Winter, W. P. (1967), *Science 158*, 1638.
Ouchterlony, O. (1958), *Progr. Allergy 5*, 2.
Pétra, P. H., and Neurath, H. (1969), *Biochemistry 8*, 2466.
Prahl, J. W., and Neurath, H. (1966), *Biochemistry 5*, 2131.
Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.
Smith, E. L., Markland, F. S., and Glazer, A. N. (1970), *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., New York, N. Y., Academic Press, p 160.
Smith, R. L., and Shaw, E. (1969), *J. Biol. Chem. 244*, 4704.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Tokoro, Y., Eisen, A. Z., and Jeffrey, J. J. (1972), *Biochim. Biophys. Acta 258*, 289.
Walsh, K., and Neurath, H. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 884.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.
Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247*, 3242.
Winter, W. P., and Neurath, H. (1970), *Biochemistry 9*, 4673.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zwilling, R., Pfleiderer, G., Sonneborn, H. H., Kraft, V., and Stucky, I. (1969), *Comp. Biochem. Physiol. 28*, 1275.

A Complex of Cytochrome *c* and Mixed Mitochondrial Phospholipids†

Kathryn M. Ivanetich, Jean J. Henderson, and Laurence S. Kaminsky*

ABSTRACT: The interaction of cytochrome *c* with mixed heart mitochondrial phospholipids has been studied as a model for cytochrome *c in vivo*. Under the conditions of our experiments, cytochrome *c* forms a complex with mixed phospholipids which is insoluble in water but can be solubilized with sodium deoxycholate. There is no preferential binding of any component of the phospholipid mixture by cytochrome *c*. The complex has an uncorrected sedimentation coefficient of 0.1 S in deoxycholate and is essentially homogeneous on ultracentrifugation and electrophoresis. Phosphorus analysis of the complex indicates that there are approximately 42 molecules of phospholipid per molecule of cytochrome *c*. The 695-nm absorbance band of ferricytochrome *c* is retaine in the complex indicating the integrity of the methionine-80–heme iron bond. Compared to cytochrome *c* the complex is stabilized against thermal denaturation in deoxycholate solution, monitored by the 695-nm absorbance band. The complex and cytochrome *c* display similar susceptibility to denaturation under conditions of high pH. There is a close correlation between solvents which extract cytochrome *c* from mitochondria and those which solubilize the complex. These results indicate the applicability of the cytochrome *c*–phospholipid complex as a model for cytochrome *c* in the mitochondrion.

Cytochrome *c* performs its function of biological electron transport in the mitochondrion where it is presumably complexed to phospholipids (Ambe and Crane, 1959; Machinist *et al.*, 1962), or to mitochondrial structural protein (Edwards and Criddle, 1966) and to cytochrome oxidase (Nicholls *et al.*, 1969).

In contrast to the other cytochrome components of the respiratory chain, cytochrome *c* is readily extractible from mitochondria with 0.15 M KCl (Jacobs and Sanadi, 1960). There has been much speculation concerning the existence of lipid–cytochrome *c* interactions in the mitochondrion and the role of lipid in the mechanism of action of cytochrome *c* (Ambe and Crane, 1959; Machinist *et al.*, 1962).

A number of attempts have been made to obtain an understanding of the environment of cytochrome *c in vivo* by studying complexes of phospholipid and cytochrome *c in vitro* (Reich and Wainio, 1961; Das and Crane, 1964; Das *et al.*, 1965; Kimelberg *et al.*, 1970; Sun and Crane, 1969; Quinn and Dawson, 1969). The majority of these studies have, however, involved complexes formed with a single phospholipid (*e.g.*, phosphatidylethanolamine) isolated from a source such as egg yolk which is not a site of action of

† From the Department of Physiology and Medical Biochemistry, University of Cape Town Medical School, Cape Town, South Africa. *Received November 27, 1972.* This work was supported by grants from the South African Medical Research Council and the University of Cape Town Staff Research Fund.