Benjamini, E., Young, J. D., Peterson, W. J., Leung, C. Y., and Shimizu, M. (1965), *Biochemistry 4*, 2081.

Cebra, J. J. (1961), *Immunology 86*, 205.

Farr, R. S. (1958), *J. Infect. Dis. 103*, 239.

Gerwing, J., and Thompson, K. (1968), *Biochemistry 7*, 3888.

Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.

Merrifield, R. B. (1964), *Biochemistry 3*, 1385.

Mitchell, B., 1839. Levy, J. G., and Nitz, R. M. (1970), *Biochemistry 9*,

Mortenson, L. E. (1964), *Biochim. Biophys. Acta 81*, 71.

Nitz, R. M., Mitchell, B., Gerwing, J., and Christensen, J. (1969), *J. Immunology 103*, 319.

Robbins, J. B., Harmovich, J., and Sela, M. (1967), *Immunochemistry 4*, 11.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Stewart, J. M., and Young, J. D. (1969), Solid Phase Peptide Synthesis, San Francisco, Calif., Freeman.

Tanaka, M., Nakashima, T., Mower, H. F., and Yasunobu, K. T. (1964), *Arch. Biochem. 105*, 570.

Young, J. D., Benjmini, E., Stewart, J. M., and Leung, C. Y. (1967), *Biochemistry 6*, 1455.

An Effect of Calcium Ions on the Activity, Heat Stability, and Structure of Trypsin*

Tibor Sipos† and Joseph R. Merkel‡

ABSTRACT: A temperature-dependent activation of trypsin by calcium was observed. The activation effect of calcium always occurred at or above the temperature optimum for the calcium-free enzyme system and was influenced by pH and the calcium concentration. In the presence of high concentrations of calcium an altered form of trypsin was stabilized which possessed greater enzyme activity than calcium-free trypsin. Addition of calcium to trypsin at pH 7.8 and 20–40° caused the development of a positive ultraviolet differential spectrum. The positive differential spectrum indicated a conformational change to a more compact structure and the formation of a calcium–enzyme complex.

Optical rotatory dispersion of trypsin in the presence of calcium showed a major conformational transition between 40 and 45° which nearly coincided with the ultraviolet differential thermal transitions and the enzyme velocity–temperature transition points. SE-Sephadex chromatography, Sephadex gel filtration, and sedimentation studies were used to show the nature of the calcium–trypsin complex and its relationship to the other components in the heterogeneous trypsin system.

It has long been known that calcium promotes the formation of active trypsin from trypsinogen (McDonald and Kunitz, 1941) and stabilizes trypsin against autolysis (Gorini, 1951; Bier and Nord, 1951). Reports in the literature (Delaage and Lazdunski, 1967) attribute this stabilization effect of calcium to the existence of a specific calcium binding site on trypsinogen which is also preserved in the active trypsin molecule. Accordingly, the binding of calcium to trypsinogen induces a conformational change which protects the molecule against the formation of inert proteins. The recent results of Abita *et al.* (1969) indicate that calcium at high concentration binds to the four N-terminal aspartyl residues on trypsinogen without inducing a structural change, and increases the affinity of the Lys^6–Ile^7 bond to trypsin hydrolysis.

Radhakrishnan *et al.* (1969) attribute the relatively slow hydrolysis rate of the Lys^6–Ile^7 bond during zymogen activation to the chemical character of the amino acid sequence in the N-terminal end of the zymogen, since the calcium effect could be abolished by partial substitution of the N-terminal aspartyl residues with glycinamide groups.

Earlier Bier and Nord (1951) showed that calcium not only protects trypsin against self-digestion, but it also slightly increased its proteolytic activity. Gorini (1951) concluded that calcium promotes the formation of a calcium–trypsin complex from a reversible inactive form.[1] Gorini also sug-

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[1] "Reversible inactive" trypsin was the designation of Kunitz and Northrop (1934) for the form of trypsin obtained by raising the temperature or by making the solution strongly alkaline. This form was in equilibrium with active trypsin ($T_a \rightleftharpoons T_i$) but if allowed to stand, unless at very low pH (2), trypsin soon irreversibly lost its activity (irreversible inactive). Evidently, if considerably ionized by either acid or base,

gested that calcium is an integral part of a more stable trypsin molecule which arises from a shift in the equilibrium toward the active form, *i.e.*, $T\cdot Ca \rightleftharpoons T + Ca^{2+}$, where T·Ca is the active calcium–trypsin complex and T is a reversible form of inactive trypsin. Gorini showed that, particularly in the absence of Ca^{2+}, T was converted into T′ which is an irreversibly inactive form of trypsin. Since the conversion of $T \rightarrow T'$ was greatly affected by temperature, presumably the irreversibly inactive form was completely denatured trypsin. This form of trypsin (T′) was readily hydrolyzed by active trypsin (T·Ca).

Green and Neurath (1953) proposed a slight modification of Gorini's scheme to account for their observation that calcium was not obligatory for tryptic activity. Their scheme assumed that active trypsin exists in a reversible equilibrium between inactive (apparently all reversible) and active forms, and calcium could combine with either form to produce an active T·Ca complex. The newly formed calcium trypsin possesses greater resistance to autolysis than trypsin without calcium.

We recently observed that calcium not only stabilized trypsin against autolysis, but also caused a temperature-dependent activation similar to that observed with a marine bacterial proteinase (Sipos and Merkel, 1968a). The phenomenon was observed in trypsin-catalyzed hydrolysis of urea-denatured hemoglobin, *p*-Tos-L-ArgOMe, and Bz-L-ArgOEt. The temperature-dependent activation of trypsin by calcium always occurred at or after the temperature optimum (transition point) for calcium-free trypsin, indicating that calcium activation required significant structural changes in the enzyme molecule.

This report describes the influence of various parameters on the temperature-dependent activation of bovine trypsin and the conformational transitions in the trypsin molecule that accompany activation.

Materials and Methods

Reagents. Bovine trypsin (two- and three-times recrystallized, salt free, Lot TRL 9FC, and TRL 100 s Lot 7JK, respectively), α-chymotrypsin (three-times recrystallized, CDI 7 CG), and hemoglobin substrate (salt free, lyophilized) were purchased from Worthington Biochemical Corp., Freehold, N. J. *p*-Tos-L-ArgOMe was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio. Bz-L-ArgOEt was purchased from Mann Research Labs. Cellulose acetate strips (Sepraphore III) were from Gelman Instrument Co., Chelsea, Mich. Active-site titrant, *p*-nitrophenyl-*p*′-guanidino benzoate, Lot K-5965, was purchased from Cyclo Chemical Co. SE-Sephadex (C-50) and Sephadex G-100 (Superfine) were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. All other chemicals used in this study were reagent quality or better.

Proteinase Activity. Proteinase estimations were made by Prescott's modification (Prescott and Willms, 1960) of the method of Anson (1938), using urea-denatured hemoglobin substrate as described before (Sipos and Merkel, 1968a). Active trypsin concentration was determined with *p*-nitrophenyl-*p*′-guanidinobenzoate according to Chase and Shaw (1967).

Esterase Activity. The spectrophotometric method of Schwert and Takenaka (1955) was used to follow Bz-L-ArgOEt hydrolysis, and the method of Hummel (1959) was used for *p*-Tos-L-ArgOMe hydrolysis as described in a previous publication (Sipos and Merkel, 1968a). For more recent studies, we followed enzyme hydrolysis of *p*-Tos-L-ArgOMe by pH-Stat measurements at pH 8.0 with a Radiometer Model TTT-11 automatic titrating equipment coupled with a type ABU 12 autoburet.

Zone Electrophoresis. Electrophoresis was carried out in a Gelman apparatus on cellulose acetate strips (Sepraphore III, No. B-755, B-759, and 1155), and using Tris or phosphate buffers under the following conditions: 0.2 M Tris buffer in the pH range of 6 to 9 and a potential of 70–90 V with 15–20 mA for 2.5–3 hr. The distribution of the protein bands along the cellulose acetate strip was determined by staining the strip with ponceau S dye (National Aniline Division, Allied Chemical Corp., New York, N. Y.) at a concentration of 0.4 g/100 ml in 5% trichloroacetic acid. The distribution of the proteinases on the Sepraphore strips was determined by overlaying the strips on chromoprotein plates, followed by incubation at 25° (Merkel, 1966). After 20–60-min incubation, proteolytically active enzyme bands became visible because the chromoproteins, phycocyanin and allophycocyanin, lost their color upon digestion. The stained Sepraphore strip and the incubated chromoprotein plate were scanned with a double-beam recording and integrating densitometer (Chromoscan, Joyce, Loebl and Co., Ltd., England). The comparison of the stained protein bands with the proteolytically active bands on chromoprotein provided a method for the determination and identification of the number of proteolytically active enzymes.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in a Canalco disc electrophoretic apparatus, using 7.5% polyacrylamide gel at pH 4.5 according to the method of Williams and Reisfeld (1964). The distribution of the protein bands was determined by staining the gel with aniline blue black as described by Davis (1964).

Chromatography of Bovine Trypsin on SE-Sephadex (C-50). Separation of bovine trypsin into the various components was accomplished by the method of Schroeder and Shaw (1968) using 0.1 M Tris-HCl, pH 7.1, with 0.02 M $CaCl_2$ at 4° without benzamidine·HCl. The SE-Sephadex column (gel height 1.8 × 85 cm) was loaded with 150 mg of trypsin (in 15 ml), and fractions of 9.0 ml were collected with a Büchler fraction collector into tubes containing 0.5 ml of 1.25 M potassium formate, pH 2.9. Protein concentration of the effluent was measured at 280 mμ and the activity of selected fractions was determined by pH-Stat titration using *p*-Tos-L-ArgOMe as substrate. The peak fractions (tubes 70–85 and tubes 92–110 in Figure 5a) were combined, reprecipitated with 70% ammonium sulfate, redissolved in 0.001 M HCl, dialyzed against 0.001 M HCl overnight, and then lyophilized. The lyophilized samples were used for *p*-nitrophenyl-*p*′-guanidinobenzoate titrations and gel electrophoresis. During dialysis 10–20% losses in specific activities were repeatedly observed.

Gel Filtration Experiments on Polydextran (Sephadex) Columns. Gel filtrations were carried out at 4° with Sephadex G-100 (Superfine) (Pharmacia Fine Chemicals, Piscataway,

trypsin occurs in a reversible denatured form (Anson and Mirsky, 1934). Presumably the reversible inactive form also exists in the intermediate pH range where trypsin is catalytically active.

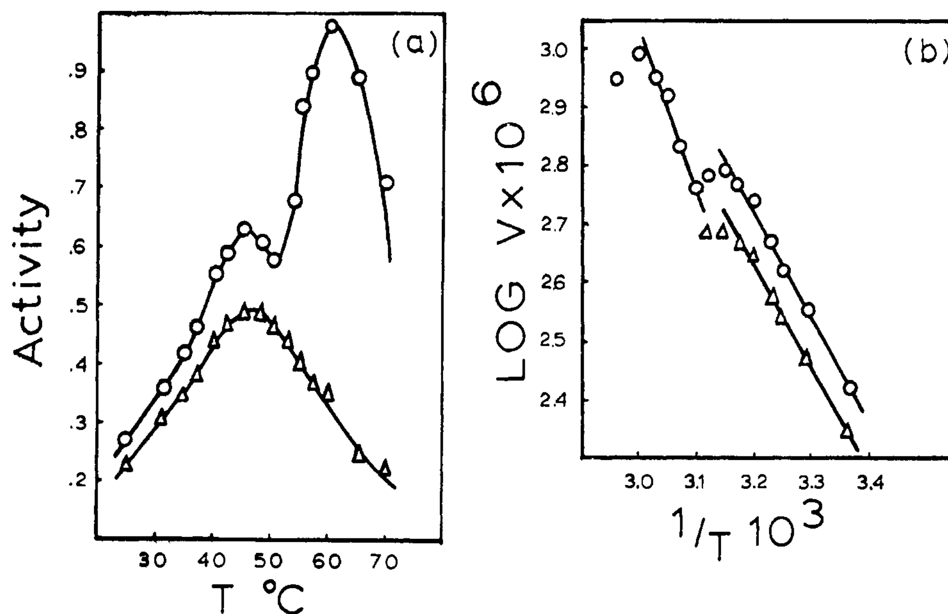


FIGURE 1: Effect of temperature. (a) Temperature-dependent activation of trypsin by calcium. All determinations were made at pH 8.0 as described for esterase activities in Materials and Methods. Trypsin concentration was 6.3 μg/ml. Activity is expressed in millimoles hydrolyzed per minute per mg of trypsin: (Δ) trypsin activity in the absence of calcium; (O) activity in the presence of 0.02 M $CaCl_2$. (b) Arrhenius plot of the trypsin-catalyzed hydrolysis of *p*-Tos-L-ArgOMe. (Δ) Without calcium; (O) with 0.02 M $CaCl_2$; V = initial velocity in moles hydrolyzed/min/mg of trypsin; T = absolute temperature.

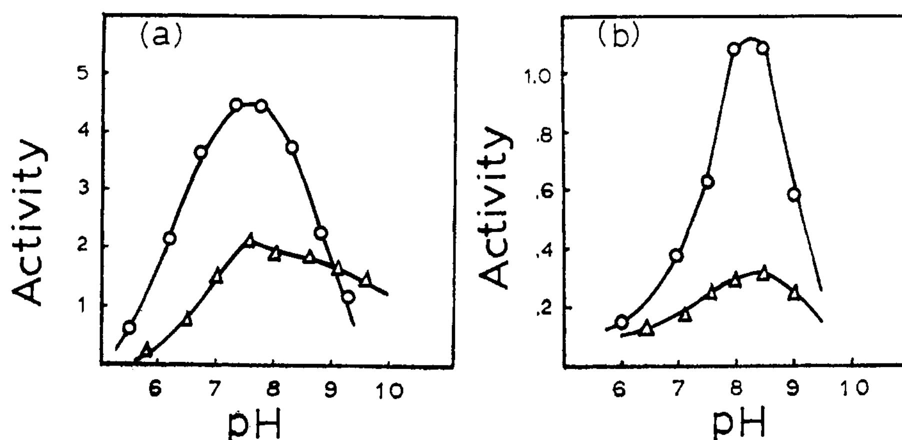


FIGURE 2: pH dependence of the calcium activation of trypsin. (Δ) Without calcium, (O) with 0.02 M $CaCl_2$; (a) 6.3 μg/ml of trypsin, urea-denatured hemoglobin substrate in phosphate buffer and activity determined at 40°; (b) 6.3 μg/ml of trypsin, *p*-Tos-L-ArgOMe substrate in Tris buffer, activity determined at 60° and expressed as millimoles of *p*-Tos-L-ArgOMe hydrolyzed/min per mg of trypsin.

N. J.) in 1.9 × 120 cm columns with 0.1 M NaCl in 0.05 M Tris buffer, pH 7.5. The Sephadex gel was allowed to swell for 2 days in distilled water, washed five times with the starting buffer, and then was packed by gravity into a 1.9 × 120 cm column (gel height 113 cm). Flow rates were approximately 12 ml/hr; 3-ml fractions were collected with a Büchler automatic fraction collector, and protein concentrations were determined by measuring the absorbance of each fraction at 280 mμ in 1.0-cm cuvets.

During Sephadex gel filtrations all conditions were kept uniform, and the same column was used in the successive experiments.

Ultraviolet Differential Spectra Measurements. Ultraviolet differential absorption spectra were measured in a Beckman DK-2A ratio recording spectrophotometer. Determinations were made on a solution containing approximately 4 mg/ml of trypsin dissolved in 0.001 M HCl. Exact aliquots of this solution were pipetted into matched quartz cells to which 1.0 ml of 0.2 M Tris buffer, pH 7.8, was added and placed into the sample and reference compartments of the spectrophotometer. Calcium chloride (0.5 ml, 1.0 M) was added to the sample cell, and the same amount of 3.0 M sodium chloride was added to the reference cell. After mixing, the spectrum was immediately recorded at 20° from 320 to 240 mμ. Thermal difference spectra were obtained by recording the difference in absorption between a sample cuvet maintained at a desired temperature for 4 min against a control cuvet kept at 20°. Ultraviolet differential spectra were also

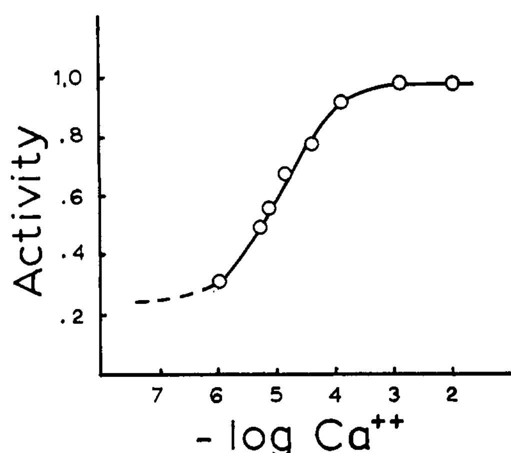


FIGURE 3: Concentration dependence of the calcium activation at 60°. Velocity in millimoles of *p*-Tos-L-ArgOMe hydrolyzed/min per mg of trypsin is plotted against the calcium concentration of the reaction mixture.

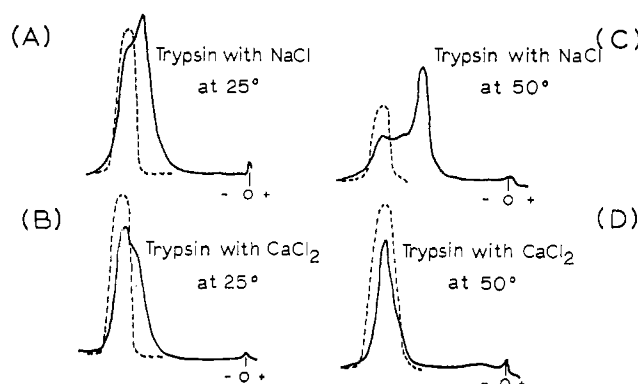


FIGURE 4: Zone electrophoresis patterns of heat-treated trypsin in the presence and absence of calcium ions. The enzyme solutions were exposed to the indicated temperatures for 3 min, immediately cooled to 4° and 15–20 μl applied to a cellulose acetate strip (1 × 6 in.) soaked in buffer. Electrophoresis was conducted at room temperature (22–24°) with the following conditions: 80–90 V, 15–18 mA, for 3 hr in 0.2 M Tris buffer at pH 6.95. The control solution was prepared by dissolving 20 mg of trypsin in 1 ml of 0.2 M Tris buffer, pH 6.95, containing 0.15 M NaCl. The calcium-treated trypsin contained 0.05 M $CaCl_2$ in place of NaCl. The drawings (A, B, C, D) are exact tracings of densitometer scannings: (——) ponceau S stained bands; (---) enzyme activity as measured by chromoprotein digestion.

recorded for sample cells containing NaCl and heated to various temperatures compared with a reference cell containing NaCl but kept at 20°.

Optical Rotatory Dispersion Measurements. Rotatory dispersion curves were obtained with a Durrum-Jasco ORD–UV-5 spectropolarimeter. The slit width of the instrument was automatically controlled to provide a constant light intensity throughout the spectral region of interest. A water-jacketed, 0.991-mm cell with fused-quartz windows was used in all measurements. Constant temperature was maintained by circulating water through the jacketed cell from a thermostated water bath. After each recording at a fixed temperature, the temperature of the water bath was quickly raised to another higher temperature. After 5-min equilibration at the new temperature the optical rotation was measured against the wavelength. The instrument was calibrated for optical rotatory dispersion measurements with D-10-camphorsulfonic acid as the standard. A base line was recorded before each set of experiments using a protein-free buffer blank. The concentration of trypsin was determined at 280 mμ, using 0.694 as the extinction coefficient for converting the absorbance into milligram per milliliter concentration (Davie and Neurath, 1955). Optical rotation is expressed as specific rotation [α].

Sedimentation Studies. Sedimentation velocity measurements were made in a Beckman-Spinco Model E ultracentrifuge equipped with a schlieren optical system. Sedimentations were performed at 20°, and the photographs were taken at 8-min intervals after attaining 59,730 rpm. The enzyme concentration was 1% in 0.002 M Tris buffer with 0.2 M $CaCl_2$ or 0.6 M NaCl, pH 7.8.

Results

Temperature–Velocity Profile and Arrhenius Activation Energies of the Calcium Activation. When the influence of 0.02 M calcium ions on the enzyme activity of trypsin was tested with *p*-Tos-L-ArgOMe as the substrate at various temperatures the patterns shown in Figure 1a were obtained. The corresponding values for the Arrhenius activation energies are shown by Figure 1b. In the presence of calcium these results yielded 8.2 kcal per mole for the low, and 12.8 kcal per mole for the high-temperature forms with a characteristic break occurring at 40–45°. In the absence of calcium a value of 8.2 kcal per mole was obtained. The break in the Arrhenius plots corresponds to the characteristic shoulder observed in the enzyme velocity *vs.* temperature curves, and as will be shown later, is also in good agreement with the ultraviolet transition temperature and the optical rotatory dispersion conformational transition point.

pH Dependence of the Calcium Activation. Figure 2a shows the pH dependence of the calcium activation of trypsin at 40° with hemoglobin as the substrate. The lower curve was obtained in the absence of calcium at 30°. The activity curve shows a broad pH maximum extending from pH 7.0 to 9.5 which is in a good agreement with Northrop and Kunitz (1932) and Crewther's (1953) results.

Figure 2b illustrates the influence of pH on calcium activation of trypsin when *p*-Tos-L-ArgOMe was the substrate at 60°. Analogous results were obtained in the presence of Bz-L-ArgOEt. The activation effect of calcium is confined to a narrow pH range between 7.5 and 8.5.

Concentration Dependence of the Calcium Activation. Activation of trypsin by calcium in the range of 40–65° was found to vary with the concentration of calcium (Figure 3). The concentration dependence of the calcium activation was investigated with *p*-Tos-L-ArgOMe as the substrate at the optimum pH (8.1), 60°, and an enzyme concentration of 12.5 μg/ml. Up to 10^{-6} M, calcium had no influence on the enzymatic activity of trypsin, but, when the concentration of calcium was increased from 10^{-6} to 10^{-3} M enzyme activity increased. There was no further increase in the enzymatic activity of trypsin beyond 10^{-3} M calcium.

Effect of Metal Ions on Trypsin at 40° and at 60°. Studies on the effect of calcium concentration on enzyme activation

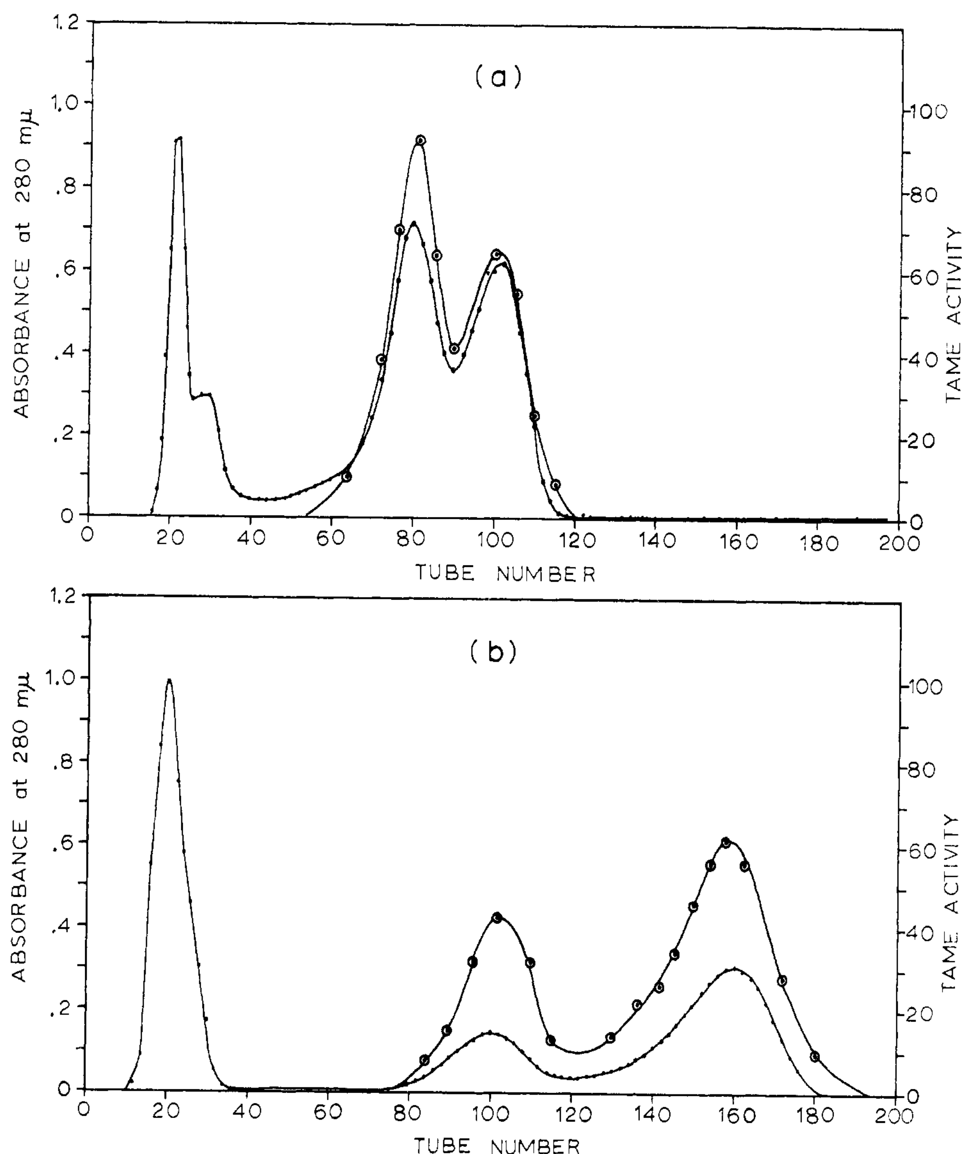


FIGURE 5: SE-Sephadex (C-50)chromatography of bovine trypsin. (a) Bovine trypsin (Worthington, twice crystallized, Lot TRL 9FC, 150 mg) was applied to the column in 15 ml of 0.1 M Tris, pH 7.1, containing 0.02 M $CaCl_2$, and eluted with the same buffer. Details of the column and procedure are given in the Materials and Methods section. (b) Bovine trypsin (same as in a) (100 mg) dissolved in 5.0 ml of 0.1 M Tris, pH 7.1, containing 0.05 M $CaCl_2$, was heated for 2.5 min at 48°. The solution was immediately cooled in an ice bath, diluted with 0.1 M Tris to a final volume of 12.5 ml (final Ca^{2+} concentration of 0.02 M), and applied to the SE-Sephadex column. In (a) and (b), ——, represents absorption at 280 mμ, -O-O-, enzyme activity in micromoles of *p*-Tos-L-ArgOMe hydrolyzed/min.

at 60° revealed that a high concentration of calcium relative to the enzyme concentration was required. This could have meant that other ions, possibly present as contaminants of calcium chloride, were responsible for the activation. To test this possibility other metal ions were tried as activators in a final concentration of 10^{-4} M. The metal ions included: Na^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}, all as chlorides. The metal ions were preincubated with the substrate (*p*-Tos-L-ArgOMe) at 60° for 5 min before the addition of (14.5 μg/ml) trypsin. Besides calcium, Mn^{2+}, Sr^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and various buffer solutions with high ionic strength also effected a temperature-dependent activation of trypsin, but to a smaller extent than calcium.

Electrophoretic Mobility of Calcium–Trypsin Complex. The above studies on the enzyme activity of trypsin under a variety of conditions suggested a calcium and trypsin interaction with the formation of a calcium–trypsin complex. Therefore, an investigation of the electrophoretic behavior of trypsin was undertaken. It was hoped that interaction with calcium would change the enzyme mobility (Nord and Bier, 1953), and reveal the presence of a calcium–trypsin complex with a higher temperature stability. Figure 4 shows the electrophoretic mobility of trypsin on cellulose acetate strips.

In the absence of calcium two peaks appeared on electrophoresis. The inactive peak predominated over the active one (Figure 4A). When trypsin was treated with calcium at 25°, a similar electrophoretic pattern was obtained, but the active band was predominant (Figure 4B). When trypsin was heated at 50° in the absence of calcium for 5 min prior to electrophoresis, trypsin was irreversibly denatured, and gave rise

to three protein bands on electrophoresis (Figure 4C). Only one of these bands showed some residual enzyme activity when it was tested by the chromoprotein method. However, when trypsin was heated in the presence of calcium under identical conditions, it gave rise to a single active band, as illustrated in Figure 4D. During the electrophoretic studies it was noted that in all cases the presence of calcium greatly increased the sharpness of the electrophoretically separated protein bands. In the absence of calcium a diffused electrophoretic pattern was obtained, which in many cases greatly obscured the interpretation of the results.

From the above results it appeared that calcium had simply caused a shift in the equilibrium between inactive and active forms of trypsin with the formation of a calcium–trypsin complex, as was suggested by previous investigators. This calcium–trypsin complex possessed greater thermal stability than trypsin without calcium. However, the temperature–activity profiles presented in Figure 1 and other evidence presented below indicate that more is involved in the reaction at raised temperatures than a simple shift in equilibrium to form active trypsin.

In contrast to the results of Nord and Bier (1953), two electrophoretically separable bands were present in our calcium-free trypsin, and the addition of calcium at room temperature only lowered the amount of inactive protein in the preparation (see Figure 4A *vs.* 4B). Heating the Ca^{2+}–trypsin solution to 50° for 3 min completely removed the inactive band. The disappearance of this band was not compensated by an equivalent increase in the active material as might be expected from the scheme of Green and Neurath (1953). Instead of an increase in activity a small loss of enzyme activity was observed.

SE-Sephadex chromatography, Sephadex gel filtration, and sedimentation studies presented below shed some light on the nature of the calcium–trypsin complex and its relationship to the other components in the heterogeneous trypsin system.

Sedimentation and Sephadex Gel Filtration Studies of Trypsin. Cunningham *et al.* (1953) showed that "when trypsin is stable, active or inactive, it exhibits the sedimentation behavior of a monodisperse solute. However, when trypsin is active and unstable it exists in a monomer–polymer equilibrium which is dependent on time and protein concentration," and in the presence of calcium, trypsin exhibits the sedimentation behavior of a monodisperse solute much like DFP-trypsin. Nord and Bier (1953) demonstrated that the aggregation is also temperature dependent.

Our temperature-dependent activation studies at first suggested the possibility of a shift in the monomer–polymer equilibrium (Cummingham *et al.*, 1953) of trypsin to the monomeric state in the presence of calcium as the principal cause of the activation effect. Sephadex gel filtration and sedimentation studies were undertaken to see how heat treatment in the presence of calcium modified trypsin. It was hoped that these studies might reveal the nature of the calcium–trypsin interaction, and provide an answer about the possibility of a monomer–polymer equilibrium of trypsin under the applied conditions.

Sephadex gel filtration studies showed that trypsin contains at least two components with slightly different elution volumes. The inactive trypsin appeared to have a higher molecular weight than the active one, and was eluted just before the active trypsin.

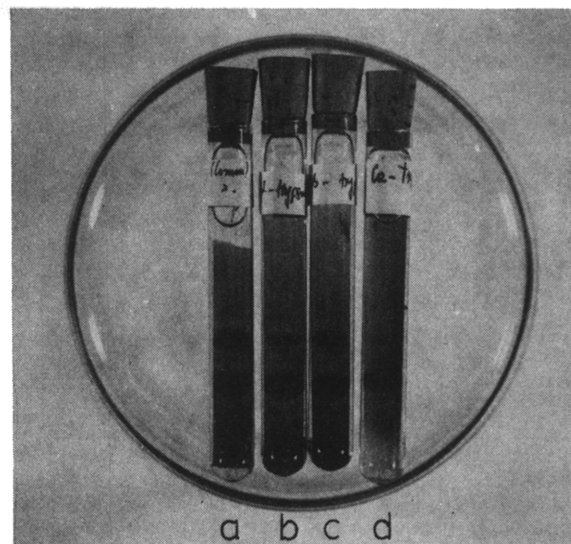


FIGURE 6: Polyacrylamide disc gel electrophoresis of SE-Sephadex (C-50) separated trypsin components. The trypsin components, obtained by SE-Sephadex chromatography were pooled, dialyzed against 0.001 M HCl overnight, and lyophilized, and 100 μg of each lyophilate was applied to the gel columns. Electrophoresis was conducted in 7.5% gel (pH 4.5) for 75 min at a constant current of 5 mA per gel tube: (a) twice-recrystallized commercial trypsin (Worthington); (b) α-trypsin (tubes 70–85, Figure 5a); (c) β-trypsin (tubes 92–110, Figure 5a); (d) T*Ca (tubes 140–170, Figure 5b).

When the heat-treated (in the presence of calcium) trypsin was subjected to gel filtration the height of the protein peak significantly decreased and a twofold increase in the specific activity was obtained. The elution pattern also revealed that during the calcium and heat treatments the inactive trypsin was hydrolyzed into smaller peptide fragments.

When the Sephadex G-100 (Superfine) column was calibrated with molecular weight markers, the active trypsin eluted with a molecular weight of 23,000. Since no higher molecular weight active components could be detected during Sephadex gel filtration of trypsin (irrespective of how trypsin was pretreated), the depolymerization idea to account for the temperature-dependent activation of trypsin by calcium was abandoned.

Preliminary sedimentation studies confirmed the Sephadex gel filtration results, and clearly showed observable differences between the sedimentation patterns of calcium-treated trypsin, and trypsin without calcium.

*Separation of T*Ca by SE-Sephadex Chromatography.* SE-Sephadex chromatographic studies of Schroeder and Shaw (1968) showed that their trypsin samples contained two active (α- and β-trypsins) and several inactive components. Later, a third component called pseudotrypsin (ψ) was isolated by Smith and Shaw (1969) from autolyzed α-trypsin. Since our electrophoretic and Sephadex gel filtration studies showed that heat treated trypsin in the presence of calcium ions was converted to a new active form (T*Ca), it seemed to be desirable to isolate and identify T*Ca by the above method.

The SE-Sephadex (C-50) elution pattern of our starting trypsin is shown in Figure 5a, which is quite similar to that of Schroeder and Shaw (1968). The calcium and heat-treated trypsin elution pattern is shown by Figure 5b.

The SE-Sephadex elution patterns of trypsin clearly show

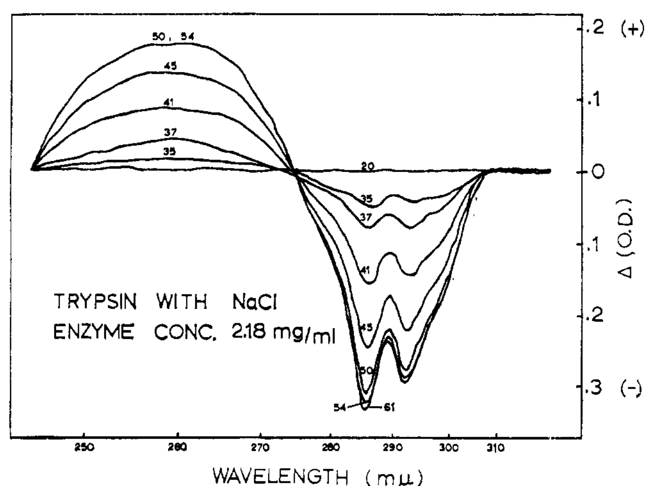


FIGURE 7: Ultraviolet differential spectra of trypsin in the presence of sodium chloride. Both sample and reference cell (20°) had identical compositions. Other conditions are described in Materials and Methods.

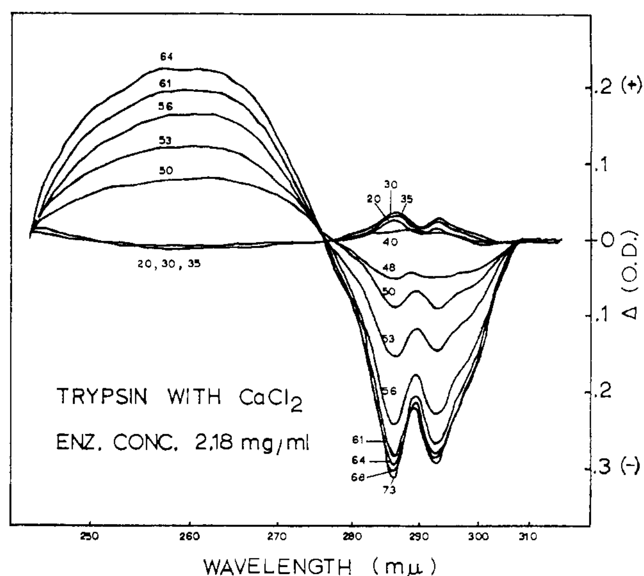


FIGURE 8: Ultraviolet differential spectra of trypsin in the presence of calcium chloride. The sample cell contained $CaCl_2$, while the reference cell contained NaCl with the same ionic strength but at 20°.

that T*Ca is distinctly different from ψ-, α-, or β-trypsins, and it can be considered as a new trypsin species. From the elution pattern in Figure 5b it appears that in the presence of calcium ions and heat, α-trypsin is converted into T*Ca. Homogeneity of the SE-Sephadex isolated T*Ca was confirmed by polyacrylamide gel electrophoresis at pH 4.5 and is shown by Figure 6.

The specific activities of the various forms of trypsin as determined by active-site titration with *p*-nitrophenyl-*p*′-guanidinobenzoate (Chase and Shaw, 1967) and by *p*-Tos-L-ArgOMe hydrolysis are summarized in Table I.

Spectral Studies of Trypsin. The differential spectra of trypsin were determined by the spectral method of Chervenka (1959). It was expected that the interaction of calcium and trypsin at elevated temperatures should give rise to a differential absorption in the 287- and 293-mμ range of tyrosine and tryptophan perturbations. The ultraviolet differential spectra of trypsin with respect to temperature in the absence of calcium ions are shown by Figure 7. The ultraviolet differential spectra in the presence of sodium chloride show that trypsin is rapidly denatured with increasing temperature and has a thermal transition temperature around 40° (50% denatured).

TABLE I: Comparative Activities of the Various Forms of Trypsin.

	Concentration by NPGB[b] (× 10^5 M)	Concentration as Determined by $OD_{280\ m\mu}$[a] (× 10^5 M)	% Active	*p*-Tos-L-ArgOMe[b]
Commercial trypsin	3.84	4.80	80.5	210
α-Trypsin	2.05	2.44	84	295
β-Trypsin	2.77	3.59	77	270
T*Ca	1.95	1.81	108	262

[a] $OD_{280\ m\mu}$ × 0.694 = mg/ml. [b] *p*-Tos-L-ArgOMe (μ/mole/min per mg). [b] NPGB = *p*-nitrophenyl-*p*′-guanidinobenzoate

The ultraviolet differential spectra of trypsin in the presence of calcium are shown by Figure 8. The presence of calcium at 20–40° caused the development of small positive differential spectra in the 287- and 293-mμ region, relative to the control (trypsin in 0.5 M NaCl at 20°). The positive differential spectra suggest a direct interaction between trypsin and calcium with the formation of a calcium–trypsin complex. Up to 45°, calcium retarded the thermal denaturation of trypsin by preventing the unfolding of the molecule when the temperature was raised. An examination of the two sets of thermal differential spectra of trypsin shows that calcium causes a 10–15° shift to higher temperatures in the thermal transition curves of trypsin. A comparison of the thermal transition curves of trypsin also shows that in the presence of calcium the enzyme has its maximum conformational stability around 20–35°. A similar result was obtained when the effect of temperature on the optical rotatory dispersion properties of trypsin was studied.

Optical Rotatory Dispersion of Trypsin. Figure 9 shows the influence of temperature on the rotatory powers of trypsin in the presence of sodium chloride at pH 7.8 and in the 210- to 280-mμ range. At 20° the rotatory powers of trypsin passed through a minimum in the range of 228- to 232-mμ ($[\alpha]_{230}$ of 1620), and had a crossover point of 215 mμ. An increase in the temperature above 35° caused the disappearance of the 210- to 220-mμ peaks and the widening of the trough at 228–232 mμ. Further increase in the temperature above 40° caused the appearance of a new trough at the 223- to 228-mμ region. When the temperature was raised to 65°, all the characteristic features of the optical dispersion curves of trypsin disappeared. These changes in the rotatory power of trypsin show the sig-

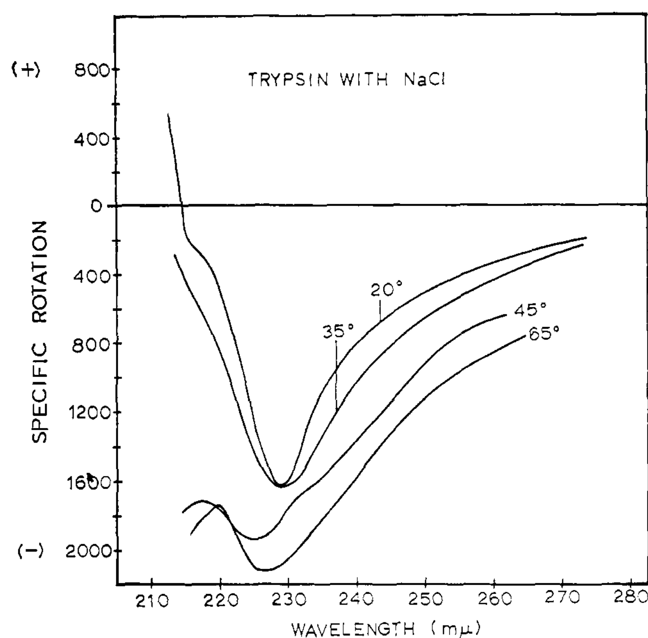


FIGURE 9: Variation of the optical rotatory power of trypsin with respect to temperature. Trypsin (5 mg) was dissolved in 3 ml of 0.002 M Tris buffer, pH 7.8, at 4° and 1 ml of 3.0 M NaCl was added. The solution was thoroughly mixed and a portion transferred with a hypodermic syringe to a jacketed optical cell. Temperature was controlled with a thermostated bath and sufficient time was allowed for temperature equilibration before each recording.

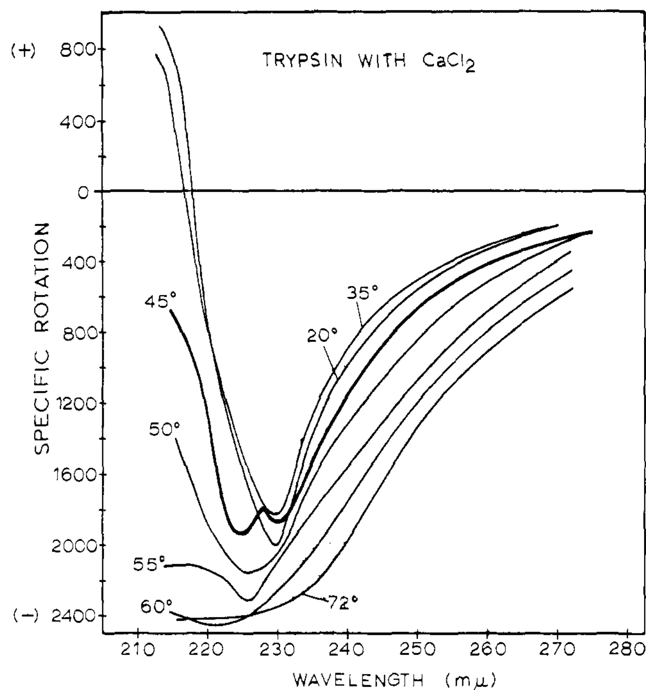


FIGURE 10: Influence of calcium on the optical rotatory power of trypsin with respect to temperature. All conditions were the same as described for Figure 9, except that 1 ml of 1.0 M $CaCl_2$ was used in place of NaCl.

nificant conformation rearrangements which take place during thermal denaturation.

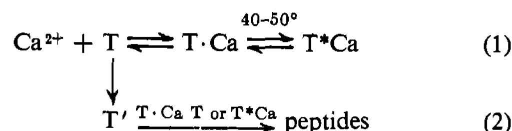
Figure 10 shows the effect of calcium on the optical rotatory dispersion of trypsin with respect to temperature at pH 7.8. The presence of calcium first caused a small deepening of the characteristic trough at 229–232 mμ, indicating a possible increase in the helical content, or some alteration in the β structure of the enzyme, *i.e.*, the formation of a more compact structure (Sage and Fasman, 1966; Yang and McCabe, 1965). A value for $[\alpha]_{230}$ of 2020, and a crossover point of 218 mμ was obtained at 20°. As the temperature increased, calcium retarded the thermal denaturation of trypsin, and permitted the recording of the conformational transitions in the range of 40–50°, where the temperature-dependent calcium activation is observed. The most important feature of the dispersion curve at 45° is the simultaneous appearance of the double trough at 229–232 mμ and at 223–226 mμ in the presence of calcium. The optical rotatory dispersion results suggest that the conformational changes in the molecular structure of trypsin are presumably responsible for the greater enzyme activity and thermal stability.

Discussion

The experimental data suggest that trypsin undergoes two distinct transitions in the presence of calcium. The first one which was observed during electrophoresis, optical rotatory dispersion, and ultraviolet studies, leads to the formation of a stabilized calcium–trypsin complex as was suggested by previous investigators (Gorini, 1951; Bier and Nord, 1951; Nord and Bier, 1953; Green and Neurath, 1953). The stabilized calcium–trypsin then hydrolytically converts the contaminating inactive component (assumed to be inactive trypsin) into small fragments (Northrop *et al.*, 1948; Gorini, 1951). An increase in the incubation temperature greatly facilitates this enzymatic reaction.

The second transition was observed while determining the temperature optimum of the trypsin-catalyzed hydrolysis of the various substrates. This transition produces a characteristic shoulder in the temperature–velocity plot when calcium ions are included in the incubation mixture. This shows that calcium is doing more than simply protecting the enzyme against heat denaturation.

The temperature-dependent transitions of trypsin in the presence of calcium and in the active pH range can be diagrammatically illustrated as



$$Ca^{2+} + T \rightleftharpoons T\cdot Ca \overset{40\text{–}50°}{\rightleftharpoons} T^{*}Ca \quad (1)$$

$$T \downarrow T' \xrightarrow{T\cdot Ca\ T\ or\ T^{*}Ca} \text{peptides} \quad (2)$$

The equations are slight modifications of those proposed by Gorini (1951). T represents the reversible active form(s) of trypsin (Anson and Mirsky, 1934; Northrop *et al.*, 1948; Schroeder and Shaw, 1968; Smith and Shaw, 1969), T′ is irreversible inactive trypsin, T·Ca is the calcium–trypsin complex, and T*Ca represents the heat-modified Ca-trypsin complex which seems to originate from α-trypsin. The scheme differs from Green and Neurath's (1953) only in that we do not show two reversible forms of calcium-free trypsin, one active and one inactive, in equilibrium, *i.e.*, $T_a \rightleftharpoons T_i$. These authors

proposed that both forms could be converted into a stabilized T·Ca but calcium was not required for enzyme activity.

In our scheme, and presumably also in Gorini's, T may represent several forms of the trypsin molecule at pH 7.8, as was demonstrated by Schroeder and Shaw (1968), which if not stabilized by formation of the T·Ca complex, readily undergo further alteration through a stepwise, temperature-dependent denaturation to finally produce irreversibly, inactive molecules (T′). The isolation of various active and inactive forms from autolyzed trypsin (ψ-trypsin, Smith and Shaw, 1969; an active trypsin with a split at Arg-105, Maroux *et al.*, 1967) support this conclusion.

Gorini showed that if calcium is removed from trypsin with EDTA at 0°, the enzyme activity is retained. However, if calcium is removed at a higher temperature (37°), the enzyme irreversibly loses its activity. This indicates that an energy-requiring transformation occurs to speed the conversion of the calcium-free structure into the final denatured form. Our conformational studies show that in proceeding from T*Ca → T·Ca → T → T′ there is progressive opening of the trypsin structure, and in the final step, the unfolded molecules readily aggregate or are enzymatically hydrolyzed unless protected by either high H^+ or OH^- concentrations. The spectrophotometric titration results of Lazdunski and Delaage (1965), Delaage and Lazdunski (1965), and our results reported here show that calcium induces a conformational change in trypsin resulting in a more compact structure(s), *i.e.*, T·Ca.

The calcium–trypsin complex(es) rapidly degrades the inactive form (T′). This step was found to be significantly accelerated by an increase in the temperature at pH 7–8. After heating for a short time (1.5–3.0 min) at 45° in the presence of 0.02 M calcium only the calcium-stabilized species remains in the solution along with the degradation products (peptides). At this higher temperature trypsin undergoes further changes in its structure with the formation of an activated calcium–trypsin complex (T*Ca) which can be isolated by SE-Sephadex chromatography. Some of the β form of trypsin persists through the heating procedure, but it appears that the α form is converted into the heat-stable T*Ca form (compare Figure 5a,b). Further characterization of T*Ca and the rates of conversion are currently under investigation and will be the subject of a future publication.

The differential spectral results suggest that the differences observed in the ultraviolet spectrum of trypsin in the presence of calcium are the result of an environmental change of the tyrosine and tryptophan chromophores. The development of a positive differential spectrum in the presence of calcium at 25–30° may be explained in the following way. Calcium reduces interactions between charged residues and causes a shift of the tyrosine and tryptophan chromophores from a polar environment (aqueous) to a nonpolar environment in the interior of the molecule. The results of Duke *et al.* (1952) and those of Delaage and Lazdunski (1965) on the titration of trypsin in the presence of calcium support these observations. According to Duke *et al.* (1952) calcium interacts with free carboxyl groups by neutralizing their charges and preventing a direct interaction of the charged carboxyl groups with the tyrosine chromophores or other available charged residues. The narrow pH optimum and the ionic concentration dependence show that the active conformation of trypsin is quite sensitive to environmental influences. The failure of Mg^{2+}, Sr^{2+}, Zn^{2+}, Co^{2+}, and other metal ions, with the exception of Mn^{2+}, to activate both the protease and esterase activity of trypsin demonstrates that calcium is the most effective ion to stabilize trypsin and also to cause a temperature-dependent activation. In the presence of calcium a shift of 10–12° in the thermal transition curves to higher temperatures tends to show that calcium somehow plays a role in the reduction of the intramolecular interactions of the charged groups in the trypsin molecule.

A comparison of the conformational transitions recorded by optical rotatory dispersion with the transition observed on the temperature–velocity plots reveals that trypsin undergoes a structural change in both cases at the same temperature range. It appears from the optical rotatory dispersion curves that the temperature transition in the molecular conformation of trypsin leads to a more disorganized state (widening of the trough at 229–233 mμ and the disappearance of the 210-to 220-mμ peaks). During this transition a short-lived intermediate must exist, which in the presence of high concentration of calcium is stabilized. The simultaneous appearance of the double trough at 223–226 mμ and at 229–232 mμ in the optical rotatory dispersion transition of the calcium treated trypsin tends to support the existence of this transitory intermediate, which in the presence of high concentration of calcium is stabilized. Additional evidence in support of the existence of a calcium-stabilized activated form of trypsin (T*Ca) is the 210- to 220-mμ rotatory peak, which is completely absent at 45° in calcium-free trypsin.

The obtained results demonstrate that in the presence of calcium at high temperatures trypsin forms an active calcium–trypsin complex which possesses greater activity and thermal stability than trypsin without calcium. It is concluded that the function of calcium during the temperature-dependent activation is to maintain a specific compact conformation of the enzyme molecules which is necessary for their catalytic activity.

The temperature-dependent calcium activation phenomenon has been observed with a bacterial proteinase, with trypsin, and also with chymotrypsin. All of these proteases are serine-type enzymes. Therefore, it is suggested that during this phenomenon common conformational forms of the enzymes with enhanced activity and higher temperature stability might be stabilized by calcium.

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References

Abita, J. P., Delaage, M., Lazdunski, M., and Savrda, J. (1969), *Eur. J. Biochem. 8*, 314.

Anson, M. L. (1938), *J. Gen. Physiol. 21*, 79.

Anson, M. L., and Mirsky, A. E. (1934), *J. Gen. Physiol. 17*, 393.

Bier, M., and Nord, F. F. (1951), *Arch. Biochem. Biophys. 33*, 320.

Chase, T., Jr., and Shaw, E. (1967), *Biochem. Biophys. Res. Commun. 29*, 508.

Chervenka, C. H. (1959), *Biochim. Biophys. Acta 31*, 85.

Crewther, W. G. (1953), *Aust. J. Biol. Sci. 6*, 597.

Cunningham, L. W., Tietze, F., Green, N. M., and Neurath, H. (1953), *Discuss. Faraday Soc. 13*, 58.

Davie, E. W., and Neurath, H. (1955), *J. Biol. Chem. 212*, 507.

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

Delaage, M., and Lazdunski, M. (1965), *Biochim. Biophys. Acta 105*, 523.

Delaage, M., and Lazdunski, M. (1967), *Biochem. Biophys. Res. Commun. 28*, 390.

Duke, J. A., Bier, M., and Nord, F. F. (1952), *Arch. Biochem. Biophys. 40*, 430.

Gorini, L. (1951), *Biochim. Biophys. Acta 7*, 318.

Green, N. M., and Neurath, H. (1953), *J. Biol. Chem. 204*, 379.

Hummel, B. C. W. (1959), *Can. J. Biochem. Physiol. 37*, 1393.

Kunitz, M., and Northrop, J. N. (1934), *J. Gen. Physiol. 17*, 591.

Lazdunski, M., and Delaage, M. (1965), *Biochim. Biophys. Acta 105*, 541.

Maroux, S., Rovery, M., and Desneulle, P. (1967), *Biochim. Biophys. Acta 140*, 377.

McDonald, M. R., and Kunitz, M. (1941), *J. Gen. Physiol. 25*, 53.

Merkel, J. R. (1966), *Anal. Biochem. 17*, 84–92.

Nord, F. F., and Bier, M. (1953), *Biochim. Biophys. Acta 12*, 56.

Northrop, J. H., and Kunitz, M. (1932), *J. Gen. Physiol. 16*, 267.

Northrop, J. H., Kunitz, M., and Herriot, R. M. (1948), Crystalline Enzymes, 2nd ed, New York, N. Y., Columbia University Press, p 140.

Prescott, J. M., and Willms, C. R. (1960), *Proc. Soc. Exp. Biol. Med. 103*, 410.

Radhakrishnan, T. M., Walsh, K. A., and Neurath, H. (1969), *Biochemistry 8*, 4020.

Sage, H. J., and Fasman, G. D. (1966), *Biochemistry 5*, 286.

Schroeder, D. D., and Shaw, E. (1968), *J. Biol. Chem. 243*, 2943.

Schwert, G. W., and Takenaka, J. (1955), *Biochim. Biophys. Acta 16*, 570.

Sipos, T., and Merkel, J. R. (1968a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 520.

Sipos, T., and Merkel, J. R. (1968b), *Biochem. Biophys. Res. Commun. 31*, 522.

Smith, R. L., and Shaw, E. (1969), *J. Biol. Chem. 244*, 4704.

Williams, D. E., and Reisfeld, R. A. (1964), *Ann. N. Y. Acad. Sci. 121*, 373.

Yang, J. T., and McCabe, W. J. (1965), *Biopolymers 3*, 209.