Alterations in the Physical and Enzymic Properties of α-Chymotrypsin in Urea and Guanidinium Chloride*

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The denaturation of α-chymotrypsin by urea and guanidinium chloride in the presence and absence of calcium chloride has been investigated by measurement of changes in absorbancy, optical rotation, and activity as a function of *p*H. Difference spectra in either denaturant were characterized by maxima at 293 and 285.5 mμ. In solutions of urea a difference peak at 231 mμ could also be observed. Constant ratios of the low- to high-wavelength difference peaks prevailed over a wide *p*H range. In either 8 M urea or 6 M guanidinium chloride the specific rotation was independent of the *p*H of denaturation and less levorotatory in the latter solvent. When calcium chloride was also present, the rotation varied with *p*H upon denaturation in urea but not in guanidinium chloride. The dependence of the rotation on *p*H was ascribed to end-group effects arising as a consequence of autolysis. Although exposure of the enzyme to 8 M urea containing calcium chloride resulted in inactivation which became increasingly irreversible as the *p*H was increased, the much more rapid inactivation that occurred in 8 M guanidinium chloride (± $CaCl_2$) was completely reversible throughout the *p*H range of 5–9.5. This finding, in conjunction with the very fast difference-absorbancy changes that occurred in this solvent, would indicate that irreversible inactivation was prevented by the rapidity of the denaturative process. The rates of absorbancy change in 8 M urea plus 0.2 M calcium ions were synchronous at all peak positions and equal to the rate of inactivation and the change in optical rotation. All reactions fitted apparent first-order kinetics. In the absence of calcium chloride the change in absorbancy at 293 and 231–232 mμ also occurred synchronously and, relative to such processes in urea containing calcium ions, was about 165-fold faster at *p*H 7. At acid *p*H values, differences in rates due to presence of calcium ions disappeared. It has been concluded that the denaturation of chymotrypsin does not involve sequential disruption of "tertiary" and "secondary" structural elements and that different regions of the molecule do not denature at different rates.

Previous publications (Martin and Frazier, 1963a,b) have dealt with the rate and extent of inactivation of α-chymotrypsin in solutions of urea and the effect of various cations, particularly calcium, on these processes. In 8 M urea, the extent of irreversible inactivation (determined after dilution at about *p*H 3) was dependent on both the initial *p*H of incubation and the enzyme concentration. An additional loss of activity could also be obtained by an unfavorable *p*H (greater than *ca.* 4) of dilution. Inactivation arising by the latter process was independent of enzyme concentration. The rates of both types of reactions were extremely rapid and maximal inactivation (irreversible) occurred at *p*H 7–7.5. Above or below this *p*H range, irreversible inactivation was less. In the denaturing environment, the enzyme was present as both reversibly and irreversibly inactivated forms with the ratio dependent on *p*H.

In the presence of calcium ions, the rate of both reversible and irreversible inactivation was suppressed in 8 M urea (Martin and Frazier, 1963b). The relation between the extent of irreversible inactivation and *p*H was sigmoid in form. At *p*H 7 and above, complete irreversible inactivation occurred; below a *p*H of about 4.5, inactivation was reversible. Strontium, but not other group IIA cations, was similar to the effect of calcium.

In this paper, the rates of change in absorbancy and optical rotation have been studied in relation to rates of inactivation of chymotrypsin in solutions of urea and guanidinium chloride in the presence and absence of calcium ions as a function of *p*H. The magnitude of such changes have also been determined.

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EXPERIMENTAL PROCEDURE

Materials.—The α-chymotrypsin preparations (lots CDI-6078, 6008, and 6013-B, 3×-crystallized, salt-free) were obtained from Worthington Biochemical Corp. Stock solutions of the enzyme were generally prepared to contain about 50 mg/ml 1 mM HCl and were kept refrigerated and used for a period of no longer than 1 week.

Urea was recrystallized as described before (Martin and Frazier, 1963a) and 10.0 M solutions were freshly prepared as needed. Guanidinium chloride was crystallized from methanol. Other materials were of reagent grade quality and used without further treatment.

All solutions were clarified by either centrifugation or filtration before use and deionized water was used throughout.

Methods.—General procedures for incubation of the enzyme in 8.0 M urea or various concentrations of guanidinium chloride and containing, as indicated, 0.20 M $CaCl_2$ at a constant *p*H and temperature (30°) have been described (Martin and Frazier, 1963a).

Rates of absorbancy change were determined with either a Cary Model 14 or a Beckman DK-1 recording spectrophotometer using the technique of differential spectrophotometry. The sample and reference cuvet contents were maintained at 30.0 ± 0.1° by means of a metal jacket through which water was circulated from a constant-temperature bath.

For relatively slow reactions, changes in absorbancy with time were determined in the following manner. At zero time, an appropriate enzyme aliquot was added to thermally-equilibrated denaturant solution (preadjusted to various *p*H values), the resulting *p*H was recorded, and an aliquot was transferred to a glass-

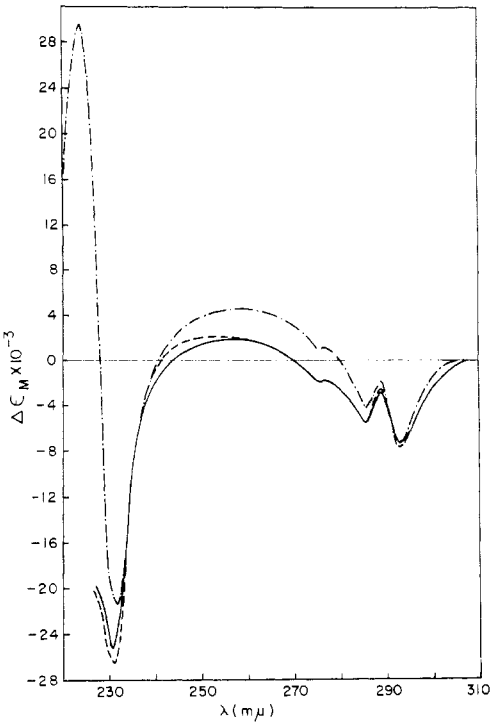

FIG. 1.—The difference spectrum of chymotrypsin after denaturation in 8 M urea containing 0.2 M $CaCl_2$ at *p*H 4.0 (————), *p*H 7.0 (– – – –), and *p*H 6.5 (·–·–·–·) versus enzyme in 0.2 M $CaCl_2$ (*p*H 6.5). All spectra have been corrected for solvent absorption and at *p*H 4.0 and 7.5 were obtained with the Cary spectrophotometer using Dynode switch position 4; slit control, 20; scan speed, 5 A/sec.; 10-mm cells; protein, 0.40 mg/ml. Data at *p*H 6.5 were obtained with the Beckman DU spectrophotometer at a protein concentration of 1.0 mg/ml in 2-mm cuvets and a constant slit width of 0.30 mm.

stoppered cuvet (quartz Ultrasil, 10-mm light path) positioned in the sample beam. Recording of the spectral change was initiated at zero time plus 0.5–1.0 minute at a chart speed of 1 in./minute.

For faster reactions, i.e., with apparent first-order rate constants greater than about 60×10^{-4} sec^{-1}, the change in absorbancy was determined by the addition of an enzyme aliquot (rapidly expressed from a calibrated syringe equipped with a Teflon needle) to a cuvet prepositioned in the sample beam of the spectrophotometer. Recording of the absorbancy change could thus be initiated at 3 seconds after zero time. At the completion of the reaction, the cuvet contents was used for the determination of *p*H. Since these reactions were complete in 15 minutes or less, any slight change in *p*H that might have occurred during the time the reaction was under observation was ignored.

The reference solution (positioned in the reference beam) for reactions conducted in denaturatant plus calcium chloride was of identical composition but minus denaturant, and the *p*H was 6.50. In the absence of $CaCl_2$, the reference solution was enzyme in 1 mM HCl (*p*H 3.3–3.5). For all rate measurements, the enzyme concentration was 0.50–1.0 mg/ml with the lowest concentration level used exclusively for readings in the region of 230 mμ.

Difference spectra were determined with either the Cary Model 14, the Beckman Model DK-1, or the Beckman Model DU spectrophotometer. In all cases, native enzyme in the nonperturbing solvent environment was positioned in the reference compartment of the dual-beam spectrophotometers. Spectral scans were made at a rate of 5 A/sec and were sufficiently slow to allow proper tracking of the slit-control mechanism in those wavelength regions (*ca.* 230 mμ) where the slit width was changing markedly with wavelength. Scans at slower speeds did not increase the values of ΔA or change the positions of wavelength maxima.

For measurements of ΔA in urea solution at about 230 mμ, the upper protein concentration was restricted to 0.40 mg/ml per 10 mm light path and, since both Lambert's and Beer's laws were adhered to at all λ_{max} positions, at least 2.0 mg/ml in a 2-mm-light-path cuvet could be tolerated. Under these conditions, differences in absorbancy were determined (Beckman DU) to be independent of slit width between 0.03 and 1.0 mm in the region of 290 mμ and between 0.09 and 0.70 mm in the 230 mμ region. At any intermediate slit width, ΔA was also independent of the next higher or lower sensitivity setting.

All difference spectra, in general, have been corrected for solvent absorption by a separate determination of the appropiate correction curve. In a few cases, the tandem-cell technique described by Herskovits and Laskowski (1962) was used. In every experiment, the same solution preparation of denaturant as used for perturbation of the protein spectrum was used for the solvent-absorption correction.

Measurements of optical rotation were made with a Zeiss 0.005° spectropolarimeter using a 10-cm tube thermostated at 30.0 ± 0.02°. Protein concentration varied between 2 and 4 mg/ml. All reported specific rotations have been corrected to their values that would obtain in water by the equation (Schellman, 1958)

$$[\alpha]_\lambda' = r_s[\alpha]_\lambda$$

where $r_s = (n_w^2 + 2)/(n_s^2 + 2)$, n_w and n_s are the refractive indices of water and solvent, respectively, and $[\alpha]_\lambda$ is the specific rotation at wavelength λ. Refractive indices were obtained with a Bausch and Lomb Model 3-L Abbe refractometer using the sodium D line. No correction was applied for the dispersion of the refractive index since Schellman (1958) has shown that in the wavelength region considered the variation in r_s would be small. At 30°, the refractive indices for the solvent systems employed were determined to be as follows: 8.0 M urea, 1.3985; 8.0 M urea plus 0.20 M $CaCl_2$, 1.4027; 6.0 M guanidinium chloride, 1.4300; 7.37 M guanidinium chloride, 1.4561; 6.0 M guanidinium chloride plus 0.20 M $CaCl_2$, 1.4383; 0.20 M $CaCl_2$, 1.3370; and water, 1.3319.

Inactivation (reversible plus irreversible) occurring under the conditions of denaturation was determined by *type A* assay (Martin and Frazier, 1963b). This involved determination of remaining enzyme activity in reaction solutions of the same composition and *p*H as the incubation solution (enzyme plus denaturant) but containing, in addition, either L-tyrosine ethyl ester at 0.0175 M (enzyme, 125 μg/ml) or *N*-acetyl-L-tyrosine ethyl ester at 0.01 M (enzyme, 40 μg/ml). The extent of irreversible inactivation was determined, after 26-fold dilution of the enzyme-denaturant solution with 1 mM HCl, in an assay system containing *N*-acetyl-L-tyrosine ethyl ester (0.01 M) and $CaCl_2$ (0.10 M) at *p*H 8.00 and 30° as previously described (*type B* assay, Martin and Frazier, 1963b).

Apparent first-order constants ($\bar{k}$, in sec^{-1}) were determined from log $(A_\infty - A_t)$ versus time plots.

Enzyme concentration was determined from absorbancy measurements at 280 mμ using a value for ϵ_M of 50,000 (Dixon and Neurath, 1957). The molecular weight was taken as 25,000.

All *p*H measurements were made at 30° with a Radiometer meter (Model TTT-1 or 22, G-202B glass and K4312 calomel electrodes) and, in the presence of organic solutes, represent apparent uncorrected values.

Results

Changes in Enzymic and Physical Properties in Urea

Difference Spectra.—The perturbation of the chymotrypsin-absorption spectrum upon denaturation in 8 M urea containing 0.2 M $CaCl_2$ is shown in Figure 1. The shape of the curve, with maxima of negative sign at 293, 285.5, and 232–231 mμ, is similar to that reported by Glazer and Smith (1961), including the maximum at 224 mμ, for alkaline-denatured chymotrypsinogen and by Chervenka (1959) for urea-denatured δ-chymotrypsin and its zymogen at the higher-wavelength region.

A difference spectrum characterized by maxima of positive sign at 292, 285, and about 234 mμ was obtained upon comparison of chymotrypsin in either 35% sucrose or 2.5 M LiCl (*p*H 3) to the enzyme in 1 mM HCl. Thus the inverted correspondence of these difference spectra to those obtained in urea is in conformity with the interpretation (Yanari and Bovey, 1960) that chromophores in apolar regions have been shifted to a more polar environment during denaturation. Refractive-index effects similar to the above results have also been reported by Glazer and Smith (1961) for glucagon and the insulin A-chain in sucrose and LiCl, and by Oppenheimer *et al.* (1963) for chymotrypsin in 20% glycerol. Measurements on the latter solution, however, extended to only about 260 mμ.

When the enzyme was denatured in 8 M urea in the absence of $CaCl_2$, spectra quantitatively similar to those in Figure 1 were obtained. For comparison, Table I lists values of $\Delta\epsilon_M$ at positions of λ_{max} for

Table I
Molar-Difference Extinction Coefficients for the Denaturation of Chymotrypsin in 8 M Urea ± 0.2 M $CaCl_2$

		$-\Delta\epsilon_M$[a]				
*p*H	$CaCl_2$	293 mμ (a)	285.5 mμ (b)	231 mμ (c)	(c/a)	(c/b)
4.0	−	7400	5500	25,200	3.40	4.57
7.0	−	7640	5550	26,500	3.46	4.76
4.0	+	7620	5130	23,900	3.14	4.67
7.0	+	7200	4750	23,400	3.25	4.92

[a] Measurements made with Cary Model 14 spectrophotometer.

denaturation of the enzyme in 8 M urea ± $CaCl_2$ at *p*H 4 and 7. The $\Delta\epsilon_M$ values at each λ_{max} position can be considered essentially constant within experimental error. Thus the constancy of the ratios of the low-to-high-wavelength changes suggests that $\Delta\epsilon_M$ values are independent of the condition of denaturation despite the marked effect that both *p*H and Ca^{2+} ions have on the extent of reversibility of inactivation (Martin and Frazier, 1963a,b).

A more thorough evaluation of the effect of *p*H on the difference-absorbancy ratios was obtained by another experiment. The infinite time values for ΔA at positions of λ_{max} were determined (Beckman DU) after incubation of chymotrypsin in 8 M urea containing 0.2 M $CaCl_2$ within the *p*H range of 3.45–8.80. The ratios of $\Delta A_{232}/\Delta A_{293}$ and $\Delta A_{232}/\Delta A_{285.5}$ were constant over the entire *p*H range and equaled (ten samples) 2.76 ± 0.11 and 4.56 ± 0.18, respectively. The absolute values for ΔA were also invariant with *p*H. As can be seen in Figure 1, the Beckman DU instrument placed the low-wavelength maximum at 232 rather than at 231 mμ as obtained with the Cary spectrophotometer. Also, ΔA at both 285.5 and 232 mμ was somewhat less. This

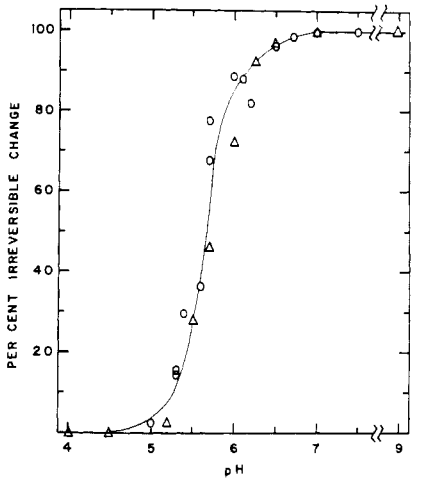

Fig. 2.—The relation between incubation *p*H and retention of ΔA_{232} upon dilution. Enzyme (1.0 mg/ml) was incubated at various *p*H levels in 8 M urea and 0.2 M $CaCl_2$ for a time more than sufficient to give maximal irreversible inactivation. Following 15-fold dilution with 1 mM HCl, ΔA_{232} was determined versus a reference solution containing enzyme and $CaCl_2$ at equivalent concentrations. Relative retention of ΔA_{232} (ΔA_{232} at pH 7.5 equal to unity) (○); relative extent of irreversible inactivation (data taken from Martin and Frazier, 1963b) (△).

explains the lower $\Delta A_{232}/\Delta A_{293}$ ratio and the essentially same $\Delta A_{232}/\Delta A_{285.5}$ ratio of the above data as compared to that listed in Table I.

Reversibility of the Difference Spectrum of Denatured Chymotrypsin.—Dilution (15-fold) of the enzyme denatured in 8 M urea plus 0.2 M $CaCl_2$ at *p*H 4.0 completely reversed the difference spectrum with the exception of a slight retention at 230–235 mμ. However, after denaturation of the enzyme at *p*H 7.5, the difference spectrum was not reversed upon dilution. These results prompted a more extensive investigation of the relation between reversibility of the difference spectrum and the extent of irreversible inactivation at various *p*H values. After incubation of chymotrypsin in the denaturant, the solutions were diluted and the difference absorbancy was measured at 232 mμ. The results are shown in Figure 2, along with data from a previous paper (Martin and Frazier, 1963b) relating the "infinite" time–irreversible inactivation to the *p*H of incubation. Similar measurements at 293 mμ showed the same parallelism between activity and absorbancy although the much smaller ΔA values precluded good quantitation over the necessary range of *p*H.

Various investigators (Chervenka, 1959; Smillie, 1959; Laskowski *et al.*, 1960; Yanari and Bovey, 1960; Oppenheimer *et al.*, 1963) have shown that a difference spectrum similar to that obtained upon denaturation can be observed upon comparison of the hydrolysis products of a protein or of a mixture of its amino acids (in the correct molar ratios) with the nonperturbed structure. It would therefore appear that retention of the difference spectrum upon dilution is the result of prior autolysis of the protein in the denaturant rather than an irreversible conformational change of the molecule. That autolysis does occur was determined by incubation of the enzyme in 8 M urea containing 0.2 M $CaCl_2$ at *p*H 6.5, with aliquots removed at various times for both the assay (*type B*) of remaining activity and the appearance of trichloroacetic acid–soluble material. The increase in irreversible inactivation closely paralleled the appearance of the latter.

In 8 M urea (no $CaCl_2$), maximal irreversible inacti-

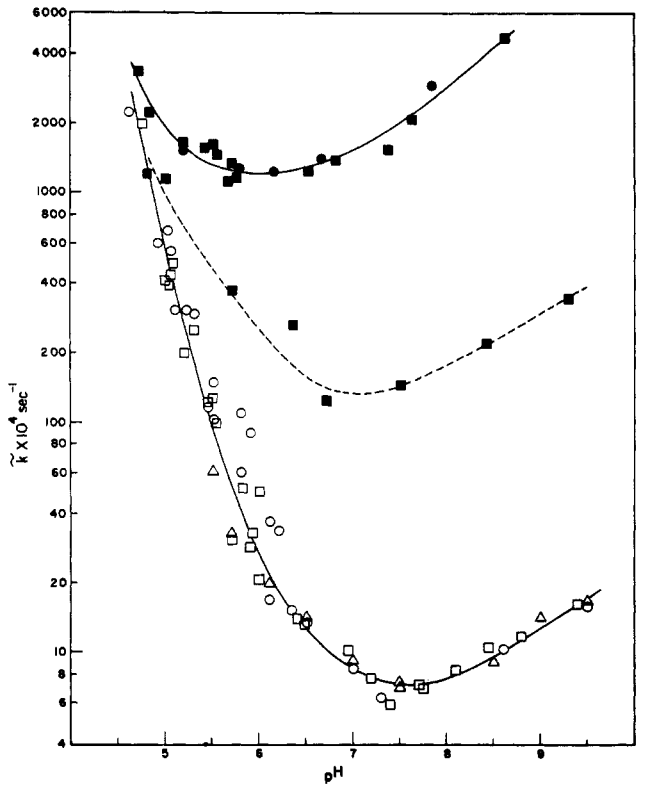

FIG. 3.—The effect of *p*H on apparent first-order rate constants for changes in ΔA_{293} (□,■), ΔA_{232} (○, ●), and activity (Δ) upon exposure of chymotrypsin (three different preparations) to 8 M urea containing 0.2 M $CaCl_2$ (open symbols) or in the absence of $CaCl_2$ (solid symbols). The dashed line represents an enzyme preparation (lot CDI-6078) which was exhaustively dialyzed against 1 mM HCl before use.

TABLE II
CONCENTRATION DEPENDENCE OF THE DIFFERENCE SPECTRUM RETAINED UPON DILUTION[a]

λ, mμ	ΔA		Ratio
	E, 1.0 (a)	*E*, 5.0 (b)	(b/a)
292	−0.050[b]	−0.083	1.66
285.5	−0.035[b]	−0.064	1.83
231	−0.235[b]	−0.398	1.69

[a] Chymotrypsin was slowly added to a urea solution maintained at *p*H 7.5 as previously described (Martin and Frazier, 1963a). Final conditions were: urea, 8 M; enzyme, 1.0 or 5.0 mg/ml. After 15-fold dilution into 1 mM HCl, spectral measurements were made using the tandem-cell technique (Beckman DK-1). [b] Actual ΔA multiplied by 5.

vation occurred at *p*H 7.5 and the per cent inactivation was logarithmically related to the enzyme concentration (Martin and Frazier, 1963a). Under similar conditions, retention of the difference spectrum upon dilution was also dependent on the concentration of enzyme (Table II). However, in contrast to the results obtained in urea plus $CaCl_2$, no parallelism existed between the extent of irreversible inactivation and the appearance of trichloroacetic acid–soluble material. For example, at *p*H 7.5 and at an enzyme concentration of 0.5 mg/ml, irreversible inactivation was 33% but only 5% of the protein was soluble in trichloroacetic acid. At 10 mg/ml, irreversible inactivation was 90% whereas only 20% of the protein was trichloroacetic acid soluble. These results suggest that irreversible inactivation in 8 M urea in the absence of $CaCl_2$ may involve only the splitting of a limited number of peptide bonds.

Dependence of the Rates of Absorbancy Change on pH.—The rate of decrease in absorbancy at 293 and 232 mμ was determined upon exposure of the enzyme to 8 M urea in the presence or absence of 0.2 M $CaCl_2$. All data fitted apparent first-order kinetics to at least 80–90% of completion, and the rate constants plotted against *p*H are given in Figure 3. In either denaturing environment, the rate of change at 293 mμ was synchronous with the rate at 232 mμ. Although not shown, more limited measurements also demonstrated that the change in absorbancy at 285.5 mμ paralleled those at the above-mentioned wavelengths.

The rate constants for denaturation in the presence of $CaCl_2$ were much smaller than those for denaturation in only urea. At acid *p*H values, however, this difference disappeared.

Rate constants obtained with an exhaustively dialyzed chymotrypsin preparation were identical to that of nondialyzed preparations in urea plus Ca^{2+} ions. In the absence of added calcium, the rate constants were lower than obtained with the undialyzed preparation (dashed line, Fig. 3). Tentatively, this would suggest that cation binding occurred during dialysis as a consequence of trace-metal contamination.

Values of the rate constants for inactivation of chymotrypsin in urea containing $CaCl_2$ are also plotted in Figure 3. As is apparent from the data, inactivation occurred at the same rate as did the absorbancy changes.

In the absence of calcium, rates of inactivation were too fast for measurement. However, the half-times involved for the absorbancy changes are in good agreement with the estimation of the time involved for complete inactivation of the enzyme in the same solution environment (Martin and Frazier, 1963a).

Optical-Rotation Studies.—The specific rotation of chymotrypsin in 0.2 M $CaCl_2$, in water, and in 8 M urea is shown in Figure 4A,B. The results are in general agreement with the data of Neurath *et al.* (1956) for the effect of *p*H on the rotation of δ-chymotrypsin (at 589 mμ) in aqueous and urea solution.

In connection with other data to be discussed it is pertinent at this point to mention that although the rotation in 8 M urea was independent of the incubation *p*H, one can estimate from previous data obtained under similar conditions (Martin and Frazier, 1963a) that, at the concentration used (4 mg/ml) for measurements, about 80% irreversible inactivation would occur at *p*H 7.5.

In 8 M urea containing 0.2 M $CaCl_2$, a minimal value for the rotation occurred at about *p*H 8 and a maximum at about *p*H 4. The *p*H dependence of the rotation in this solvent system could be owing either to the complications of autolysis (above a *p*H of 5) or, as a less likely possibility, to the survival of some structural aspect of the protein (or fragments thereof) in the denaturant system. An example of the latter phenomenon has been reported by Steiner (1964) for the denaturation of lysozyme in urea (9 M), but apparently complete disorganization was achieved in 6 M guanidinium chloride. Although the denaturation of α-chymotrypsin in guanidinium chloride will be treated more fully later on, the effect of this denaturant on the rotation of the enzyme will be described here for better comparison with the results in urea.

In 6 M guanidinium chloride, the specific rotation was independent of *p*H but *less* than that in 8 M urea, and an increase in the guanidinium chloride concentration to 7.37 M did not result in a greater levorotation (Fig. 4B). The rotation in the same denaturant containing Ca^{2+} ions is shown in Figure 4C and, relative to the results in

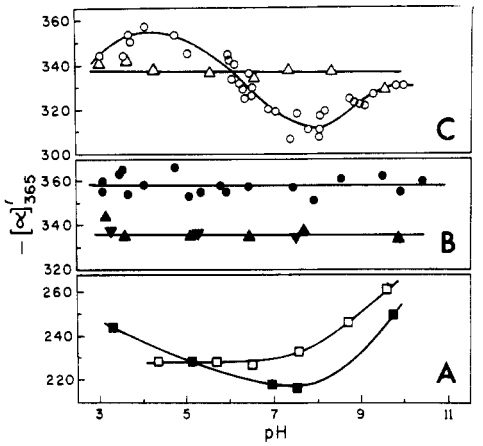

FIG. 4.—The specific rotation of chymotrypsin. (A) In water (■) and in 0.2 M $CaCl_2$ (□). (B) After denaturation in 8 M urea (●) or guanidinium chloride at 6 M (▲) and 7.37 M (▼). (C) After denaturation in 0.2 M $CaCl_2$ containing 8 M urea (O) or 6 M guanidinium chloride (△).

the corresponding urea solvent system, was more levorotatory in the *p*H range 6–9 but less so in the *p*H range 3–6.

When the enzyme was denatured in 8 M urea containing 0.2 M $CaCl_2$ at either *p*H 4 or 7.5 followed by a readjustment of the *p*H values to 7.5 and 4, respectively, the rotations were found to be dependent only on the *p*H of denaturation (Table III). When the enzyme

TABLE III
EFFECT OF *p*H ALTERATIONS AND ENZYMIC DIGESTION ON THE SPECIFIC ROTATION OF CHYMOTRYPSIN IN 8 M UREA CONTAINING 0.2 M $CaCl_2$

*p*H of Denaturation	$-[\alpha]'_{365}$
4.1	357
	358[a]
7.5	322
	322[b]
4.0	355[c]
7.5	345[c]
4.0	301[d]
7.5	287[d]

[a] After adjustment to *p*H 7.5. [b] After adjustment to *p*H 4.1. [c] Enzyme at *p*H 2.0 prior to denaturation. [d] Enzyme (*ca.* 30 mg/ml) digested with pepsin (0.16 mg/ml) at *p*H 2.0 for 16 hours, then solution *p*H raised to 8.0 and further digested with trypsin (0.14 mg/ml) for 4 hours. Temperature was 30°. Specific activity relative to enzyme control was 0.70 and about 40% of total material was soluble in trichloroacetic acid (5%).

was digested by both pepsin and trypsin prior to solution in urea and $CaCl_2$ at either *p*H 4 or 7.5, a more positive rotation was also obtained (Table III). Thus digestion of the enzyme prior to denaturation or the occurrence of autolysis (dependent on the *p*H of denaturation) can result in a decrease in levorotation.

In the last-mentioned experiment, a control sample of enzyme was kept at *p*H 2 and 2° (no decrease in specific activity) during the digestion of chymotrypsin by pepsin and trypsin, but, unexpectedly, when the sample was denatured at *p*H 7.5, the expected low levorotation (see Fig. 4C) was not obtained (Table III). Exposure of the protein to the low *p*H might have resulted in some conformational change that enabled a considerably more rapid denaturation rate to occur with the attendant prevention of autolysis.

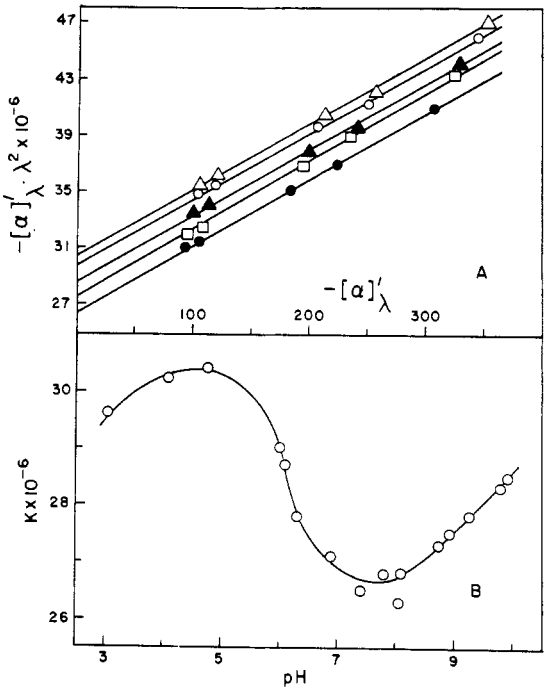

FIG. 5.—Rotatory dispersion data of chymotrypsin after denaturation in 8 M urea and 0.2 M $CaCl_2$ at *p*H 3.05 (O), 4.75 (△), 6.35 (□), 8.05 (●), and 9.94 (▲) (A); and variation in the *K* term of the Drude equation with *p*H (B) (denaturation as in A).

The dependence of the rate of denaturation on *p*H in 8 M urea containing $CaCl_2$ bears a resemblence in form to the specific rotation obtained in the same solvent between about *p*H 5 and 9 (compare Fig. 3 to Fig. 4C). If the extent of peptide-bond cleavage bears some inverse relation to the rate of denaturation, i.e., the slower the denaturation rate the greater the extent of hydrolysis of denatured molecules by remaining active ones, then a difference in the magnitude of the end-group effect, which results in a decrease in levorotation, could be responsible for the dependence of the rotation on *p*H above a *p*H of 6. As deduced from measurements of optical rotation on polyamino acids, end-group effects are largely nonexistent above a polymer length of 11–20 residues (Urnes and Doty, 1961). Assuming that this limit is valid for the present case, then up to a *p*H of about 6, wherein about 90% of the enzyme is irreversibly inactivated but the rotation is equal to or greater than in guanidinium chloride, it would follow that fragments produced by autolysis must be in excess of the above-mentioned residue length. Such considerations might also afford an explanation for the lack of a *p*H effect on the specific rotation of the enzyme denatured in only 8 M urea, providing that the extent of irreversible inactivation is owing solely to autolysis.

Optical-Rotatory-Dispersion Studies.—The variation in specific rotation with wavelength after denaturation in either urea or guanidinium chloride fitted the single-term Drude equation.

$$[\alpha]_\lambda' = \frac{K}{\lambda^2 - \lambda_c^2}$$

Representative plots (Yang and Doty, 1957) are shown in Figure 5A. In all solvent systems, λ_c was independent of *p*H, and the values with standard deviation are given in Table IV. Various workers have obtained similar results (see Urnes and Doty, 1961).

In 8 M urea and 0.2 M $CaCl_2$, $[\alpha]_\lambda'$ was dependent on the *p*H of denaturation although λ_c was constant.

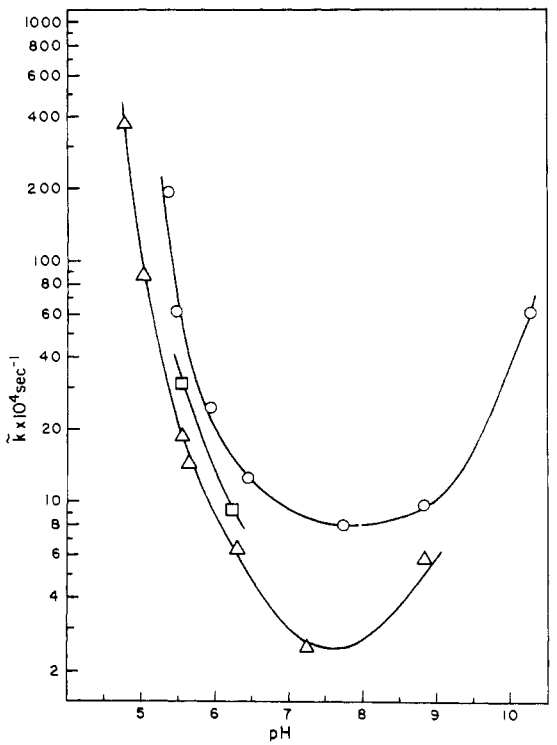

FIG. 6.—Apparent first-order rate constants for the change in optical rotation of chymotrypsin upon solution in 8 M urea containing 0.2 M $CaCl_2$ (O) and with the addition of 0.032 M 3-indolepropionate (□) or 0.022 M indole (△).

TABLE IV
EFFECT OF *p*H AND DENATURANT ON λ_c

Denaturant	$CaCl_2$ (0.2 M)	*p*H	λ_c
	+	4.35– 9.60	232.1 ± 0.9
8 M urea	−	3.05– 9.94	222.1 ± 1.1
8 M urea	+	3.10–10.4	219.7 ± 2.3
6 or 7.37 M guanidinium chloride	±	3.03– 9.55	217.3 ± 0.9

Therefore, the *K* term in the Drude equation paralleled the change in $[\alpha]_\lambda'$ (Fig. 5B; cf. Fig. 4C). Although the data are not presented, a similar parallelism between the change in $[\alpha]_\lambda'$ and *K* with *p*H also obtained for the enzyme in 0.2 M $CaCl_2$.

*Dependence of the Rate of Optical-Rotation Change on p*H.—Apparent first-order kinetics characterized the change in optical rotation at 365 mμ and the rate constants obtained over a range of *p*H are plotted in Figure 6. Comparison with the similar solution system (8 M urea plus 0.2 M $CaCl_2$) of Figure 3 reveals that changes in optical rotation, absorbancy, and activity are parallel processes.

In the absence of $CaCl_2$, the optical-rotation changes in 8 M urea were too rapid for measurement and provide suggestive evidence that they probably occur at the same rate as do the absorbancy changes under similar conditions.[1]

Indole and 3-indolepropionate reduce the rate of optical-rotation change of chymotrypsin in 8 M urea containing 0.2 M $CaCl_2$ (Fig. 6). Thus these inhibitors, presumably by interaction with the active site of the enzyme,[2] effect a decrease in the rate of denaturation over the *p*H range wherein, at least in their absence, either completely *reversible* or *irreversible* inactivation can occur. It has also been previously shown that up to a *p*H of about 7 the rate of irreversible inactivation was less than the rate of activity loss (reversible plus irreversible) of the enzyme in the denaturing environment and that indole and 3-indolepropionate decreased the rate of at least the former process (Martin and Frazier, 1963b). From this and other evidence already presented it is therefore believed that the rate constants obtained for changes in either absorbancy or optical rotation describe the time-course changes of the denaturative process.

Spectral and Activity Changes in Guanidinium Chloride

The irreversible inactivation of chymotrypsin in guanidinium chloride plus 0.2 M $CaCl_2$ at *p*H 6.5 is shown in Table V. At a denaturant concentration of 3.5–4 M, the loss in activity was very rapid and extensive. At concentrations greater than 6 M, full regeneration of activity was achieved by dilution.

Determination of the rate of inactivation in 4 M guanidinium chloride by both *type A* (i.e., in the presence of denaturant) and *B* (i.e., after dilution) assay procedures at *p*H 6.5 showed that inactivation (reversible plus irreversible) in the denaturant was faster than the rate of irreversible inactivation. Thus a reversible (initially) denaturation process is followed by a reaction or series of reactions (hydrolysis of denatured protein by remaining native enzyme) which lead to an irreversible state.

When the enzyme was added to a solution at *p*H 6.5 containing L-tyrosine ethyl ester in the presence or absence of 4 M guanidinium chloride, the same initial velocity was obtained. The substrate thus protects the enzyme against denaturation and subsequent autolysis.

From *p*H 5.0 to 9.5, incubation of chymotrypsin in 8 M guanidinium chloride for 10 minutes followed by dilution into 1 mM HCl yielded complete recovery of activity. Similar results were obtained in the presence of Ca^{2+} ions.

The difference spectrum resulting from solution of chymotrypsin in 6 M guanidinium chloride, in the presence or absence of $CaCl_2$ and at both *p*H 4 and 7, was similar to that obtained upon urea denaturation. Maxima were at 293 and 285 mμ. The spectral region below 270 mμ was inaccessible even with cuvets of 2-mm light path, owing to the high absorption of the denaturant. The molar-difference extinction coefficients were somewhat less than obtained in urea (Table VI).

The rate of absorbancy change at 293 mμ in 6 M guanidinium chloride containing 0.2 M $CaCl_2$ at *p*H 6.5 was apparent first order with time ($\tilde{k}$, 1660 × 10^{-4} sec^{-1}) to better than 90% of completion. At *p*H 4.7 the reaction was very much faster and exceeded our methods for rate measurements. The rapidity of the change in absorbancy at 293 mμ would be in harmony with the fact that under similar conditions the rate of inactivation was too rapid for measurement. It appears reasonable to assume, then, that both processes occur simultaneously.

DISCUSSION

The rate of absorbancy decrease parallels the change in optical rotation upon exposure of chymotrypsin to

[1] In a previous communication (Martin, 1961) it was erroneously reported that the rate of optical-rotation change of the enzyme (determined at a single *p*H with a Rudolph Precision polarimeter) was the same in 8 M urea in either the presence or absence of $CaCl_2$.

[2] In the same solution environment, the rate of optical-rotation change of DIP-chymotrypsin and chymotrypsinogen was not affected by the presence of indole (unpublished results, C. J. Martin).

TABLE V
EFFECT OF GUANIDINIUM CHLORIDE CONCENTRATION ON THE IRREVERSIBLE INACTIVATION OF CHYMOTRYPSIN AT *p*H 6.5 IN THE PRESENCE OF 0.2 M $CaCl_2$

Time (min)	A/A_0[a] in Guanidinium Chloride at (M)							
	3.0	3.5	4.0	4.7	5.3	6.0	7.0	8.0
2	0.98	0.73	0.33	0.43	0.80	1.11	1.15	0.96
4		0.69	0.15	0.43				0.94
6	0.82	0.38	0.09			1.00	1.08	
10	0.67	0.20	0.06	0.41	0.79	0.96	1.06	0.96

[a] Relative activity left (*type B* assay).

urea over a wide *p*H range and occurs simultaneously with the loss in activity. Viscosity changes are very much slower (Martin, 1961). One can thus conclude that disruption of the protein does not proceed in a manner that permits changes in absorbancy, optical rotation, and catalytic activity to reflect sequential reactions involving aspects of the structure whose change would be most intimately related to these methods of measurement. Also, since apparent first-order kinetics were observed in all cases with no evidence of biphasic curves, as observed for the thermal denaturation of ribonuclease (Scott and Scheraga, 1963) or for the urea denaturation of δ-chymotrypsin,[3] different regions of the molecule probably do not denature at different rates. Neurath *et al.* (1956) have shown that changes in optical rotation closely paralleled inactivation of δ-chymotrypsin in 7 M urea at *p*H 3.5 and 0°. Changes in various physical properties have also been shown to proceed concomitantly upon denaturation of ribonuclease (Nelson and Hummel, 1962) and lysozyme (Steiner, 1964) in urea.

In the presence of calcium ions, the rate of absorbancy, optical rotation, and activity changes were markedly decreased during urea denaturation. This, coupled with the observation that the disparity in rates largely disappeared at a low *p*H, strongly implicates a stabilization against conformational changes by calcium binding at higher *p*H values (see Martin and Frazier, 1963b for further discussion). Chervenka (1960) has also observed that calcium decreased the rate of absorbancy change of chymotrypsinogen upon solution in urea.

In the wavelength region usually investigated, maxima in the difference spectra of proteins are attributed to tryptophan in the region of 293 mμ (Donovan *et al.*, 1958), to tyrosine in the 285–288 and 277–280 mμ region (Laskowski *et al.*, 1956; Scheraga, 1957; Wetlaufer *et al.*, 1958), plus a contribution by tryptophan and to the benzene chromophore of phenylalanine in the spectral range of 250–270 mμ (Donovan *et al.*, 1958). Various authors (Yanari and Bovey, 1960; Bigelow and Geschwind, 1960; Leach and Scheraga, 1960) have discussed the factors, e.g., medium and charge effects, denaturation, etc., which give rise to the difference spectra of proteins in these wavelength regions.

Imahori and Tanaka (1959) and Rosenheck and Doty (1961) have shown that the helix-coil transition of several synthetic polypeptides gives rise to a hypochromic effect in the region of 190 mμ and a hyperchromic effect in the *ca.* 230-mμ region. Glazer and Smith (1960, 1961) have reported that a difference peak at this latter wavelength also attends the denaturation of proteins. The interpretation has therefore been made (Glazer and Smith, 1961; Rosenheck and Doty, 1961) that a difference maximum at about 230 mμ is associated with an alteration in the backbone structure of proteins, i.e., a conformation-dependent change in the absorptivity of the peptide bond. It has been emphasized, however, that the low-wavelength peak cannot necessarily be attributed to a singularly unique event and various possible contributions to the phenomenon have been discussed by Glazer and Smith (1961) and Wetlaufer (1962). In particular, Wetlaufer (1962) has emphasized that perturbation of the tyrosyl and tryptophyl chromophores should be expected to make strong contributions to ΔA in this spectral region.

Recently, Eisenberg and Edsall (1963) have shown that the ratio of $\Delta A_{236}/\Delta A_{287}$ remains constant for human serum albumin subjected to a variety of denaturation conditions. They concluded, therefore, that the low-wavelength peak for this protein had its origins in the environment of the tyrosine residues and that contributions from other groups or conformation effects would appear to make a negligible contribution to the difference spectrum.

As reported in this paper, constant ratios of the low- to high-wavelength difference maxima were obtained upon exposure of chymotrypsin to urea containing $CaCl_2$ over a *p*H range that resulted in either reversible or irreversible denaturation. In the absence of $CaCl_2$, constant ratios were also obtained at the two *p*H values (4 and 7) investigated. As one possibility, it could be considered that the maximum at about 230 mμ reflects only the perturbation of the tryptophan and tyrosine (plus phenylalanine) spectrum. However, a similar result would also be obtained if the low-wavelength peak contained contributions from various chromophores, including that arising from a change in peptide-bond absorptivity, and equally normalized at all *p*H values. Unfortunately, the occurrence of autolytic reactions complicates interpretation but results recently obtained for the urea denaturation of DIP-chymotrypsin indicate that the magnitude of the difference peak at about 230 mμ may not have its origins solely in an altered environment of the aromatic amino acid residues.[4]

Within the above context, the rate of spectral changes at *ca.* 230 mμ and at 285–293 mμ during the denaturation of proteins might be expected to be observed as either nonparallel or synchronous events. The urea denaturation of chymotrypsin would represent an example of the latter case. The former has been reported to occur (changes at 232.5 mμ, slower) during the denaturation of ovalbumin in either guanidinium chloride or in urea (Glazer and Smith, 1961). However, in

[3] Results to be published, C. J. Martin.

[4] When DIP-chymotrypsin (prepared from lot CDI-6078 α-chymotrypsin) was denatured in 8 M urea containing 0.2 M $CaCl_2$ at *p*H 7, the $\Delta A_{231}/\Delta A_{293}$ and $\Delta A_{231}/\Delta A_{285.5}$ ratios were considerably lower (three separate experiments) than those that obtained for denaturation at *p*H 4. The values could be increased to that which obtained for the *p*H 4 denatured solution by adjustment of the *p*H 7 denatured solution to *p*H 4. The magnitude of the *p*H 4 denatured solution spectral changes was not affected by raising the *p*H to 7. However, another DIP-chymotrypsin preparation, prepared from a different lot number of chymotrypsin, showed no such differences. Further experiments are in progress.

TABLE VI
MOLAR-DIFFERENCE EXTINCTION COEFFICIENTS FOR THE DENATURATION OF CHYMOTRYPSIN IN 6 M GUANIDINIUM CHLORIDE

pH	$CaCl_2$ (0.2 M)	$-\Delta\epsilon_M$[a] 293 mμ	$-\Delta\epsilon_M$[a] 285.5 mμ
4.0	−	4850	2650
7.0	−	5100	3000
4.0	+	6200	4700
7.0	+	6800	4600

[a] Measurements made with Cary Model 14 spectrophotometer.

a repeat of their experiments the same rate constant was found to describe the spectral changes of ovalbumin in 8 M urea at both the high- and low-wavelength regions (Martin, 1964). In contrast, Scott and Scheraga (1963), in a careful study of the kinetics of the thermal denaturation of ribonuclease, demonstrated that the rate of change in absorbancy at 235 mμ was slower than the spectral change at 287 mμ. They emphasized, however, that this was not to be interpreted as indicating two singular events but rather two aspects, i.e., a change in the perturbation of the tyrosyl chromophores in addition to a change in peptide-bond absorptivity, of the same general phenomenon. It was also shown that both the change at 287 and at 235 mμ was composed of a slow and a fast reaction indicating that parts of the molecule possibly denature at differing rates. Whether one can further extrapolate this data to imply that contributions to the low-wavelength peak elicited by conformational changes not seen at 287 mμ also reflect, in part, breakdown of the secondary structure remains to be seen.

Both Foss (1961) and Scheraga (1961) have used a helical structure relieved by random regions as a model for protein denaturation. On the basis of this model it was considered that a probable sequence of events during denaturation would be that which involved disruption of first the tertiary structure and then the secondary structure. Other possibilities were not excluded.

Experimentally, the thermal transition of ribonuclease at several *p*H values has been found to be equal by both spectrophotometric and polarimetric measurements (Hermans and Scheraga, 1961). For lysozyme at *p*H 2.2, the transition temperature measured spectrophotometrically was about 25° lower than the polarimetrically determined transition (Foss, 1961). Foss therefore concluded that a sequential disruption of tertiary and then secondary structure was probable.

The spectrophotometric (292 mμ) and polarimetric transition temperatures for chymotrypsin at *p*H 2 are also unequal with the former about 2° lower than the latter (Havsteen *et al.*, 1963). However, although a change in optical rotation is generally equated with an alteration in the secondary structure of a protein (Urnes and Doty, 1961), a change in solvation with attendant changes in position of vicinal groups in the region of asymmetrical carbon atoms should also contribute to the optical rotation (Tanford, 1962). This effect would also contribute to the formation of the difference spectrum. Thus the concept that changes in absorbancy and optical rotation reflect changes in the "tertiary" and "secondary" structure of a protein, respectively, is undoubtedly an oversimplification. Both types of measurement "see" various, but not completely overlapping, facets of the same general phenomenon.

On the basis of the results of Havsteen *et al.*, (1963) (*vide supra*), and of the possible contribution of a conformational change in the peptide-backbone structure to the difference maximum in the 230 mμ region, the transition temperature for the absorbancy change at this wavelength would possibly be different from that determined by spectral changes due to perturbation of tyrosyl and tryptophyl groups. This implicitly assumes that a change in peptide bond absorptivity would also contribute largely to a change in optical rotation. Results obtained with chymotrypsin at *p*H 2, however, do not show such a result. The transition temperatures, determined from measurements at all peak positions (292, 285, and 231 mμ), were the same (*ca.* 33°) within experimental error.[5] Obviously, further work is necessary to clarify the interpretation of differences in transition temperatures detected by different physical methods and the origin and significance of the difference maximum in the 230 mμ region of the spectrum.

Guanidinium chloride is generally conceded to be a more effective denaturant than is urea (see Gordon and Jencks, 1963). The results of this paper would in general conform to this interpretation. In 6–8 M guanidinium chloride, not only are the spectral changes much faster than in urea but irreversible inactivation does not occur. The prevention of autolysis most probably is prevented by the great rapidity of the denaturative process.

The specific rotation of chymotrypsin (in the presence of Ca^{2+} ions up to a *p*H of about 6) and the molar difference extinction coefficients in guanidinium chloride are somewhat lower than those obtaining in urea solvent systems. Whether this can be considered evidence for a greater disorganization of the protein in urea, however, is doubtful. Certainly the low value for λ_c would imply otherwise. Furthermore, the characteristics of the denaturant itself would be expected to alter not only the degree of solvation but also both spectral and optical rotation measurements through refractive index and vicinal group effects.

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Reactions of Seleno- and Sulfoamino Acids with Hydroperoxides*

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This research was undertaken to explore the reactions of seleno- and sulfoamino acids with hydroperoxides because of their relevance to the mechanisms of selenium functions in animal biochemistry. Reactions of methionine, cystine, and their selenium analogs with hydrogen peroxide and several organic peroxides have been studied. Methionine reacts most rapidly with hydrogen peroxide, but selenocystine causes the most extensive decomposition. Both methionine and selenocystine react much more slowly with organic hydroperoxides. Chemical data on the intermediates produced suggest that under certain conditions the reactions may be catalytic. The chief conclusion is that selenocystine has the chemical capacity to act as an antioxidant by the reduction of peroxides.

Vitamin E and dietary selenium elicit a similar nutritional response in many animal species. Since vitamin E is primarily a lipid antioxidant, selenium may perform the same function. Several factors must be evaluated when considering an antioxygenic role for selenium: (1) It is metabolized in the same manner as sulfur; (2) only minute quantities of dietary selenium are required to elicit a biological response. In view of its dilution by corresponding sulfur compounds, the question arises how selenium compounds can produce such dramatic results. The answer probably lies in their relative reactivity, such that a small concentration of a selenium compound is equivalent to a higher concentration of its sulfur analog.

Little comparison has been made of the reactivity of biologically important sulfur and selenium compounds. But several workers (Dennison and Condit, 1949; Woodbridge, 1959) report that alkyl selenides and diselenides are more efficient oil antioxidants and peroxide decomposers than their sulfur analogs. Since peroxides are the chief products of lipid peroxidation, a comparison of their reactions with some biological sulfides and selenides is appropriate. Accordingly, this paper reports a study of the action of sulfo- and seleno-amino acids on hydrogen peroxide and several organic peroxides.

Methods and Materials

Materials.—Selenocystine was prepared in good yield from sodium benzyl selenide and α-amino-β-chloropropionic acid methyl ester according to the directions of Painter (1947). Several workers (private communications) report difficulty in the synthesis of benzyl selenomercaptan. We were able to obtain this compound in 20–25% yield, if experimental conditions were carefully controlled. Air must be rigorously excluded. The reaction vessel was a 3-necked, 3-liter flask, equipped with a mechanical stirrer (center neck) and 3-way standard taper adapters (side necks). One adapter was fitted with (1) a condenser and $CaCl_2$—Drierite tube and (2) a small round-bottomed flask with rubber-tubing connection and a screw clamp. Selenium powder was stored in the flask until time for its addition. The other adapter was fitted with (1) a dropping funnel for admission of benzyl chloride and (2) an inlet for nitrogen gas. This apparatus made it possible to flush the system continuously with nitrogen and to exclude air until the latter stages of the preparation. Fractional distillation of the impure product gave a fraction, bp 115° (32 mm), which was used for subsequent reactions. Painter reported a bp of 102° (20 mm) and a best yield of 45%. All other reactions were carried out as he described with results comparable to his. Both selenocystine and selenomethionine are now commercially available from the Cyclo Chemical Co., Los Angeles, Calif.

Cystine disulfoxide was synthesized according to the method of Emiliozzi and Pichat (1959). Other reagent-grade chemicals were from commercial sources and satisfactory for use without further purification.

Methods.—The solubility and stability of selenocystine and cystine required the use of aqueous acid in all determinations. The following acids were used:

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