treatment with fresh enzyme does not change this situation significantly. Thus it appears that susceptible peptide bonds in a long polypeptide chain may be less susceptible when that chain becomes reduced in length or when distant residues determining the specificity are cleaved from the chain. In this connection it is probably of some significance that each of the two leucine residues in glucagon is adjacent to a tyrosine or tryptophan residue. Thus the susceptibility of these bonds to chymotrypsin B may be due to the close proximity of these residues.

Although chymotrypsin α does not cleave the leucine bonds of glucagon, it is known to do so in a number of other proteins including the B-chain of insulin (Sanger and Tuppy, 1951), ovine pituitary adrenocorticotrophic hormone (Li *et al.*, 1955), bovine ribonuclease (Hirs *et al.*, 1956), and human hemoglobin (Konigsberg and Hill, 1962). There is at the present time no obvious common pattern to the sequences about these susceptible bonds.

It is possible that chymotrypsin B may prove useful as an additional tool for sequence studies of polypeptides and proteins. If a lesser specificity of the enzyme is corroborated by studies on other polypeptide chains, it may be valuable for the further controlled reduction in size of the larger tryptic and α-chymotryptic peptides.

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The Hydrogen Ion Equilibria of Chymotrypsinogen and α-Chymotrypsin*

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The titration curves of chymotrypsinogen and α-chymotrypsin in water, 20% formol, and 8 M urea have been determined. The curves for chymotrypsinogen are consistent with its reported amino acid content. The curves for α-chymotrypsin, however, indicate an excess of one group over that which would be expected from the amino acid content. The group is cationic, titrates in the extreme alkaline region, and may be tentatively identified as a guanidino residue. No evidence for abnormal carboxyl groups in chymotrypsinogen or α-chymotrypsin were found.

Mechanisms for the catalytic action of α-chymotrypsin have been proposed which use the existing independent amino acid side chains (Bruice, 1961; Balls, 1962) while others utilize various covalently bonded structures as the active site. (Bernhard, 1959; Rydon, 1958; Stewart, 1963). Since a common feature of the sites proposed is that they have some ionic character, it seemed reasonable to expect that some of these proposals might be excluded on the basis of the ionic equilibria of the native enzyme as compared to the monoacyl enzyme. It also seemed likely that one could obtain information about the "active center" in the enzyme by comparing the titration curves of the enzyme with its precursor.

The titration curve of α-chymotrypsin from *p*H 3 to 9 was determined by Kunitz (1938). Herriott (1954) indicated that α-chymotrypsin gained a basic residue on activation of chymotrypsinogen and that this new group could not be accounted for by the known amino acid composition (Keil *et al.*, 1962). Erlanger (1960) proposed that this might be a guanidino group with an abnormally low *pK*. Chymotrypsinogen has also been titrated (Wilcox, 1958) but the data have not appeared. Since no detailed quantitative analysis of the titration curve was possible, this work was undertaken to provide sufficiently precise data to determine what differences, if any, might exist between α-chymotrypsin and chymotrypsinogen. Such titration curves might also prove useful for a study of α-chymotrypsin and its monoacyl derivatives directed toward defining the composition of its active site and to gain an insight into its mechanism of action.

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Experimental

Materials.—Chymotrypsinogen (Armour lots K021-125-1 and K0210109-2) was the generous gift of Dr. E. Sampsa. Both samples had been recrystallized twice from ammonium sulfate and twice from ethanol. α-Chymotrypsin (Armour lots of K-147173, 143, K021-114-2, K02-275, and K021-221-2) was the gift of Mr.

TABLE I

TITRATION OF 4 ML 0.15 *M* KCl WITH 1 *N* HCl AT 20°

*p*H Observed	1 N HCl Added (divisions)[a]	Total Volume (divisions)[a]	Normality ($H^+ \times 10^3$)	$-\log (H^+)$	$-\log f^{(b)}$	f
7	—	11560	—	—		
4.00	2	11562	0.17	3.762	0.238	0.58
3.75	3	11563	0.25	3.586	0.164	0.68
3.50	5	11565	0.43	3.364	0.136	0.73
3.25	$8^1/_2$	11569	0.74	3.134	0.116	0.77
3.00	15	11575	1.29	2.887	0.113	0.77
2.75	26	11586	2.24	2.646	0.101	0.79
2.50	46	11606	3.96	2.402	0.098	0.78
2.25	83	11643	7.13	2.147	0.103	0.79
2.00	148	11708	12.64	1.898	0.102	0.79
1.90	185	11745	15.75	1.803	0.097	0.78
1.80	235	11795	19.92	1.701	0.099	0.78
1.70	299	11859	25.12	1.599	0.101	0.79
1.60	388	11948	32.47	1.489	0.111	0.77
1.50	487	12047	40.42	1.393	0.107	0.78

[a] Each division represents 0.346 μl. [b] $-\log f = p\text{H} + \log (H^+)$.

Charles Damaskus. All preparations were recrystallized from ammonium sulfate or ethanol.

All other materials were reagent grade. Distilled water was used throughout.

Preparation of Protein.—Protein solutions were passed through a mixed-bed ion-exchange column in the chloride form [Rexyn IRG 501 (H-Cl)], 0.6 meq/g wet wt, Fisher Scientific Company. The weight ratio of resin to protein was approximately 20:1; this ratio gave the same values as a weight ratio of 50:1. The salt-free protein was collected and the solution was brought to the proper concentration of salt by the addition of 3.0 M KCl. The final *p*H of these solutions varied from 3.5 to 4.5 within the *p*H range where the proteins are stable. Such treatment gave results which were similar to those obtained from extensively dialyzed material, with the advantages of speed and more manageable volumes.

Protein concentrations for both zymogen and enzyme were obtained from the relationship $E^{1\%}_{280m\mu} = 20.0$ (Wilcox *et al.*, 1957) on suitable dilutions. All readings were made on a Zeiss Spectrophotometer. The molecular weight of chymotrypsinogen was taken as 25,000 and that of chymotrypsin was taken as 24,600, calculated from the amino acid analysis summarized by Keil *et al.* (1962). Ordinarily, 5% solutions of the proteins in 0.3 M KCl were prepared and used within an 8-hour period. By diluting this solution 1:1 the final concentration of protein was 2.5% in 0.15 M KCl.

Titration.—Standardization of the *p*H scale for the *p*HM-4C meter (Radiometer-Copenhagen) with G-202B glass electrode and K-100 calomel open junction reference electrode, by means of a series of standard buffers, was found to give an undesirable variation at the extreme ends of the scale. To avoid these variations, the scale was standardized by means of the known activity coefficients of the hydrogen and hydroxyl ions in a system similar to that used in the present study. Levy (1958) found the activity coefficient for 0.1 M KCl solutions at 20° to be 0.88 while Harned and Owens (1950) reported 0.783. For 0.15 M KCl solutions we find the activity coefficient to be 0.79 (Table I) while the interpolated value from Harned and Owens is 0.77. This difference therefore would require a correction of our *p*H values by the addition of 0.01 unit to bring the values reported here to correspond to the *p*H scale derived without liquid junction.

For the alkaline curve, the values for the titration of 4 ml 0.15 M KCl with 1 N KOH are given in Table II. It can be seen that the electrode system apparently has no potassium error and that the activity of the hydroxyl ion may be assumed to be one for this system (Levy, 1958). Standardization of the scale can be made by adding with a microburet, the exact calculated amount of 1 N HCl needed to bring 4 ml 0.15 M KCl to *p*H 2.00. The meter is adjusted to read *p*H 2.00. The alkaline portion is adjusted to *p*H 12.50 by the addition of 535 divisions (185.3 μl) 1.0 N KOH to this solution. If the *p*H 2.00 point is correctly adjusted, *p*H 12.50 may be obtained with a variation of less than 1% of the total base added. The scale, standardized in this manner, gave correct readings for a series of standard buffers and permitted reproducible results for the titration of the various proteins tested.

TABLE II

TITRATION OF 4 ML 0.15 M KCl WITH 1 N KOH AT 20°

*p*H Observed	1 N KOH Added (divisions)[a]	Total Volume (divisions)	Normality ($OH^- \times 10^3$)	$-\log (OH^-)$	*p*H $-\log (OH^-)$[b]
7.00	—	11856	—	—	—
10.50	7	11862	0.59	3.231	13.73
10.75	12	11868	1.01	2.995	13.74
11.00	16	11872	1.35	2.870	13.87
11.25	25	11881	2.10	2.676	13.94
11.50	42	11898	3.53	2.452	13.95
11.75	70	11926	5.87	2.231	13.98
12.00	122	11978	10.19	1.992	13.99
12.25	214	12070	17.73	1.751	14.00
12.50	387	12243	31.62	1.500	14.00

[a] Each division represents 0.346 μl. [b] $p\text{H} + p\text{OH} = pK_w$.

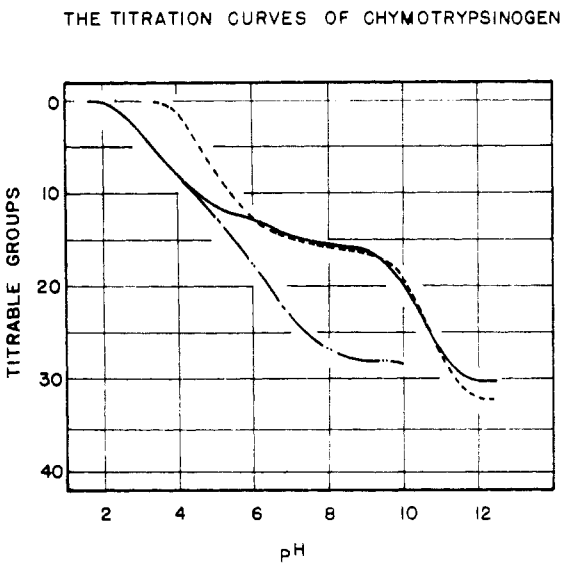

FIG. 1.—The titration curves of chymotrypsinogen. The solid line is the curve for chymotrypsinogen in 0.15 M KCl at 20°. The broken curve is the titration in 20% formol and the dashed curve is the titration in 8 M urea–0.15 M KCl.

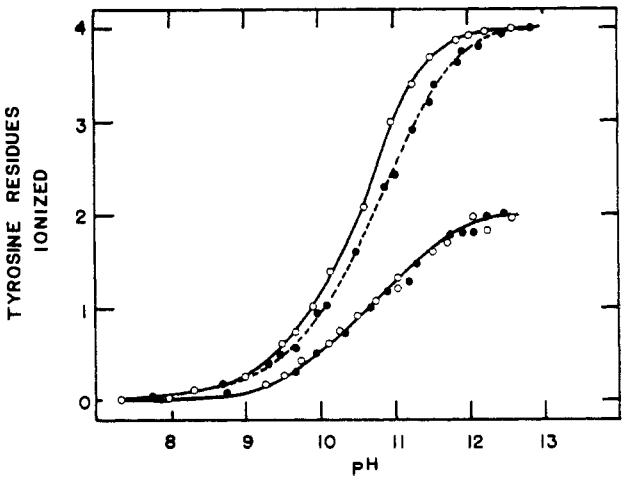

FIG. 2.—The spectrophotometric titration curves of tyrosine in chymotrypsinogen and α-chymotrypsin. The solid circles are the curves obtained for α-chymotrypsin; the open circles are the curve for chymotrypsinogen. In 0.15 M KCl (lower curve) both α-chymotrypsin and chymotrypsinogen give the same curve. In 8 M urea–0.15 M KCl (upper curves) the α-chymotrypsin is the dashed curve and chymotrypsinogen is the solid curve.

The standardization was performed before and after each protein titration and was found to be reproducible. All titrations reported were continuous. Blank corrections for volume change during the protein titration were made by multiplying the normality at each *p*H (see Tables I and II) by the total volume at that *p*H; the resulting blank value was subtracted from the protein titration curve.

Spectrophotometric Titrations.—Spectrophotometric titrations of chymotrypsinogen and α-chymotrypsin in 0.15 M KCl and in 8 M urea–0.15 M KCl were carried out at 20° ± 0.2° in a Cary Model 14 spectrophotometer with the 0–0.1 scale expansion. The concentration of protein was 2×10^{-5} M. A solution of the protein was titrated with 1 M KOH to the desired *p*H and then scanned. Values from *p*H 7 to 9.5 were difficult to maintain even when precautions to exclude CO_2 were taken. In this range, however, very little difference could be detected. For *p*H values greater than 9.5 the *p*H was constant as measured before and after the scan in the *p*HM-4C *p*H meter. The calculation of ionizable tyrosine groups was made by the method of Havsteen and Hess (1962), using a molecular extinction of 2400 for the ionized tyrosine in 0.15 M KCl and a molecular extinction of 2150 for tyrosine in 8 M urea.

Because the changes in optical density were reported to be time dependent (Chervenka, 1960), the titration was also run by adding 1 ml of 0.01 M Tris-HCl buffer at the proper *p*H to 2 ml of 0.15 M KCl containing 1.5 mg protein and scanned within 3 minutes. The results by this method were essentially the same as those from the continuous method.

TABLE III
NUMBER OF IONIZABLE GROUPS PER MOLE

	Chymotrypsinogen		α-Chymotrypsin	
	Amino Acid Content		Amino Acid Content	
	Titrimetric	Analytical	Titrimetric	Analytical
Carboxyl	12	12	14	14
Imidazole	2[a]	2	2[a]	2
α-Amino	1	1	3	3
ε-Amino	13[b]	13	13[b]	13
Phenolic	2(4)[c]	4	2(4)[c]	4
Guanidino	4[b]	4	3(?)[b]	3

[a] Estimated from the break in the mid-point of the titration curves. [b] The method for obtaining these figures is discussed in the text. [c] From the spectrophotometric titrations in 8 M urea–0.15 M KCl.

RESULTS

Chymotrypsinogen.—The hydrogen ion–binding curves for chymotrypsinogen in 0.15 M KCl, 0.15 M KCl–20% formalin, and in 0.15 M KCl–8 M urea are shown in Figure 1. The curve in 0.15 M KCl is reversible. The reversibility was determined by titrating with base to *p*H 12.5, adding acid to *p*H 2.0, and retitrating with base to *p*H 12.5. This latter titration is useful in estimating the number of groups liberated by hydrolysis (Marini and Wunsch, 1963). Preparations of chymotrypsinogen from various sources had sufficient proteolytic activity to make the retitration essential for quantitation.

The titration curve of chymotrypsinogen was reversible from *p*H 1.5 to *p*H 12.6. No change in the curves could be seen when solutions were allowed to stand at either extreme for as long as 1 minute. Prolonged standing at low *p*H, however, caused an irreversible gelation at *p*H 4. Nevertheless, that portion of the curve from *p*H 2.00 to *p*H 4.00 was identical with that shown in Figure 1. While accurate measurements were not possible in the *p*H range 2.0–1.5, titration of the protein solution from *p*H 2.00 to *p*H 1.50 with acid gave values which were only 0.2 equivalent higher than the blank. Since this is within the limit of error in this region it was assumed that the maximum binding capacity for both proteins was obtained at *p*H 2.00. An analysis of the titration curves of chymotrypsinogen (Fig. 1) shows an excellent agreement with the amino acid content which also indicates that no groups were ionized at *p*H 2.

Analysis of the curve was performed by the procedures outlined by Edsall and Wyman (1958) and by Levy (1958), and the results are shown in Table III. The carboxyl groups may be assumed to titrate between *p*H 2 and *p*H 5.5. In this region 12 groups are titrated. The mid-region from *p*H 5.5 to 8.5 repre-

sents the titration of the α-amino groups and imidazoyl groups and the curve shows that 3 groups are titrated. Titration from *p*H 8.5 to 12.5 will detect the ε-amino groups and phenolic groups. In this range 15 groups are titrated. For the *p*H range 2–12.5 a total of 30 ionizable residues can be titrated in chymotrypsinogen (12 carboxyl groups, 3 imidazoyl and/or α-amino groups, and 15 ε-amino and/or tyrosine phenolic groups). The designation of each region and its particular ionizing residue is facilitated by an examination of the formol titration curve.

Formaldehyde reacts with the uncharged amino and imidazoyl groups and makes them more acidic. In water 12 groups are titrated from *p*H 2 to 5.5, while in formaldehyde (broken curve, Fig. 1) 15 groups are titrated. The α-amino and imidazoyl groups which titrate at a higher *p*H in 0.15 M KCl are made more acidic by formaldehyde. Since 15 groups are titrated at *p*H 5.5 in formaldehyde while only 12 are titrable in 0.15 M KCl, we assign 12 carboxyl groups titrating in the *p*H 2–5.5 region. The 3 groups titrating between *p*H 5.5 and 8.5 are the α-amino and imidazoyl groups. From the break in the mid-region of the curve there appear to be 2 groups of one species. From the amino acid analysis, the mid-region is divided into 2 imidazoyl groups and 1 α-amino group.

In formaldehyde the carboxylic acid groups and the imidazoyl and α-amino groups have been titrated at *p*H 5.5 therefore it is evident that only ε-amino groups and phenolic groups remain to be titrated. Since phenolic groups are not ionized at *p*H 8.5, only the ε-amino groups may be titrated in formaldehyde between *p*H 5.5 and 8.5 and, from the curve, 13 ε-amino groups are present.

The additional groups titrating in water are phenolic groups. Evidence has been presented for the ionization of 2 tyrosine residues (Wilcox, 1958) in chymotrypsinogen. This has been confirmed (Fig. 2). With the 2 tyrosine groups, the total number of ionizable residues in chymotrypsinogen is 30 in the *p*H range from 2 to 12.5.

Ionization of the guanidino groups is apparently out of the range of the titration since a definite leveling of the curve is seen between *p*H 12.0 and 12.5. The arginine residues may be estimated, however, from the isoelectric point. Passage of chymotrypsinogen through a mixed-bed resin (H-OH) gave the salt-free protein with *p*H 9.66. This value can be corrected for charges on the protein due to hydroxyl ion concentration to *p*H 9.71, which is the isoelectric point. At this point the negative charges on the protein are equal to the positive charges. The number of groups titrated from the point of maximum proton binding to the isoelectric point is equal to the total number of cationic centers.

The isoelectric point found here is somewhat higher than the 9.5 reported by Anderson and Alberty (1948) but would be in agreement with a theoretical isoelectric point of 10 calculated from the titration curve. Assuming an isoelectric point of 10, an excellent agreement between the titrimetric and the analytical data can be shown. At *p*H 10, 20 of the 30 titrable residues have been titrated. If 12 carboxyl groups have been titrated as seen from the formaldehyde titration, then there are 12 negative charges on the protein at *p*H 10.0 and there must be 12 positive charges to balance the carboxyl groups. These twelve cationic centers are composed of ε-amino groups and guanidino groups. From the curves it can be seen that there are 10 groups left to titrate to *p*H 12.5. Of these two are phenolic groups which are uncharged in the un-ionized condition. Therefore there are 8 ε-amino groups with positive charges and there must be 4 guanidino groups making a total of 12 positive residues. The total cationic centers are 2 imidazoyl groups, 1 α-amino group, 13 ε-amino groups, and 4 guanidino groups.

This calculation ignores the contribution of negative charges due to tyrosine phenolic groups which at *p*H 10 gives rise to 0.5 equivalents. This means that, of the 10 groups remaining to be titrated, only 1.5 are phenolic and 8.5 are ε-amino groups. The condition at the isoelectric point is therefore 12.5 negative charges due to 12 carboxyl groups and 0.5 phenolic groups and this is balanced by 12.5 positive charges (8.5 ε-amino groups and 4 guanidino groups).

In 8 M urea, the analysis of the curve is the same. The *pK* of acetic acid in 7 M urea is raised from 4.78 to 5.28 (Levy, 1958). Chervenka (1961) reports a similar increase for the carboxyl group of glycine in 7.9 M urea. If the upper limit of the carboxylate titration is shifted from *p*H 5.5 to 6.0 in 8 M urea, then the number of carboxylic acid groups in urea is 12.3 as it was in 0.15 M KCl. The α-amino groups and imidazoyl groups may be estimated by assuming them to titrate up to *p*H 8.5. Between *p*H 6.0 and 8.5, 3.8 groups are titrated. This probably indicates an overlap with the ε-amino group titration. From *p*H 8.5 to 13.5 there are 16 groups titrated. Since four of these are phenolic groups (Fig. 2), 12 ε-amino groups are titrated. This confirms the analysis derived from the 0.15 M KCl titration.

The isoelectric point of chymotrypsinogen in 8 M urea is 9.8 at which point 19.6 groups have been titrated. At this *p*H, in urea, the chymotrypsinogen has 12 negative charges from the carboxyl groups which must be balanced by 12 positive charges from the ε-amino residues and the guanidino groups. Of the 12 groups remaining to be titrated from *p*H 9.8 to 13.5, 4 are tyrosine phenolic groups and 8 are ε-amino groups. The remaining 4 cationic groups must be guanidino groups which are not titrable in 8 M urea even though the titration has been carried out to *p*H 13.5 (see Fig. 4).

α-Chymotrypsin.—An examination of the curve obtained in 0.15 M KCl reveals a total of 35 ionizable residues (Fig. 3). This is in excess of the 34 residues expected from the activation of chymotrypsinogen. Rovery *et al.* (1957) showed that chymotrypsinogen gains 2 α-amino groups and 2 carboxyl groups while losing 1 guanidino residue. This represents a net gain

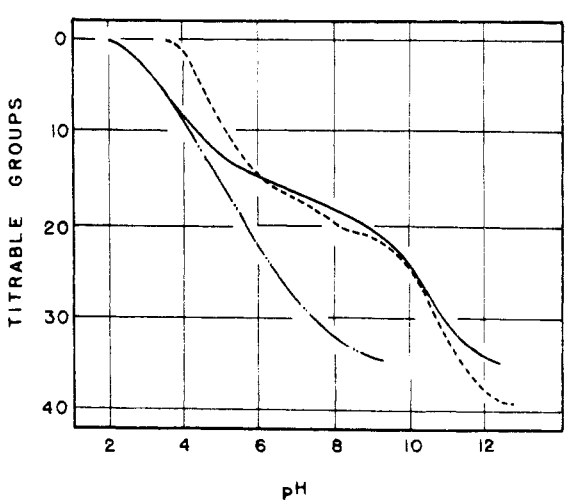

FIG. 3.—The titration curves of α-chymotrypsin. The solid line is the curve for α-chymotrypsin in 0.15 M KCl at 20°, corrected for autolysis. The broken curve is the titration in 20% formol and the dashed curve is the titration in 8 M urea–0.15 M KCl.

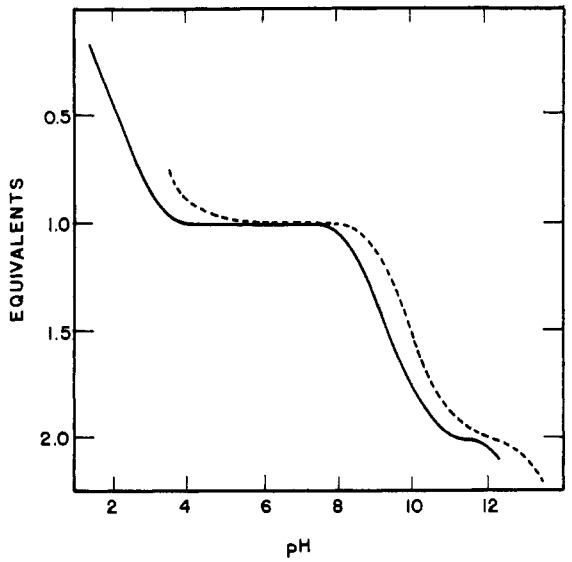

FIG. 4.—The titration curves of arginine in 0.15 M KCl (solid curve) and in 8 M urea–0.15 M KCl (dashed curve).

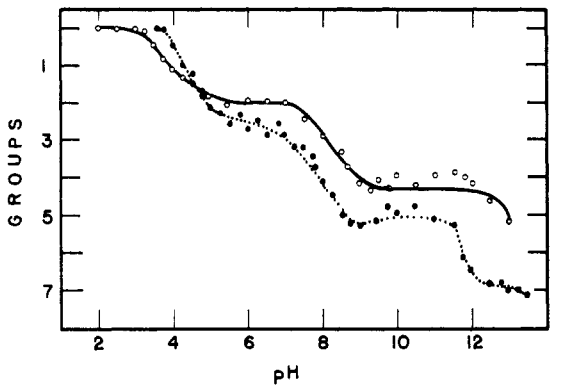

FIG. 5.—The difference of the titration curves of α-chymotrypsin and chymotrypsinogen. The method for obtaining these curves is discussed in the text. The solid curve is the difference in 0.15 M KCl; the dashed curve is the difference in 8 M urea–0.15 M KCl.

of 4 ionizable residues in the range studied. If the curve is arbitrarily partitioned by assigning *p*H 5.5 as the limit of the carboxylate titration and *p*H 8.5 as the limit of the α-amino and imidazoyl titration, as was the case with chymotrypsinogen, then there are 14 carboxylic acid groups and 5.2 α-amino and imidazoyl groups. From the amino acid content (Table III) there are 14 carboxyl groups and 5 α-amino and imidazoyl groups. This is confirmed by the addition of formaldehyde at *p*H 5.5, which shows that 5 additional groups become titrable. The interpretation of the portion of the curve from *p*H 2.0 to *p*H 8.5 is consistent with the known amino acid content.

Since there are 14 carboxyl groups, and 5 α-amino and/or imidazoyl groups, the remainder of the curve must be due to 13 ε-amino groups and phenolic tyrosine groups. From the chymotrypsinogen titration and from amino acid analysis there are 13 ε-amino groups. From the spectrophotometric titration (Fig. 2) there are 2 ionizable tyrosine residues for a total of 34 ionizable residues. Since the curve indicates 35 residues, an excess of 1 group occurs in this region. Because the curve does not reach a plateau in the *p*H 12 region, the excess group titrating in this region may be tentatively assigned to the guanidino group ionization.

The curve in the region above *p*H 8.5 is difficult to analyze because the isoionic point of α-chymotrypsin is not an experimentally determinable parameter. Attempts to obtain the isoionic point by passage of α-chymotrypsin through the mixed-bed ion-exchange resin were unsuccessful because of autolysis of the enzyme. Values greater than *p*H 7.6 could not be obtained although the isoelectric point determined by electrophoresis has been reported to be 8.2 (Anderson and Alberty, 1948; Kubachi *et al.*, 1949). While α-chymotrypsin must have an isoionic point less than that of chymotrypsinogen because it has one less guanidino group, it is unreasonable to expect a change of 2 *p*H units. Assuming that the cationic sites for α-chymotrypsin are normal, the isoelectric point will occur at that *p*H where there are 14 positive charges left to balance the 14 negative charges due to the ionized carboxyl groups. This point will occur when 21 groups have been titrated (14 carboxyl groups, 5 α-amino and/or imidazoyl groups, and 2 ε-amino groups). From the curves the isoelectric point is *p*H 9.25. At this point the groups remaining to be titrated are 11 ε-amino groups, 2 phenolic groups, and the guanidino groups.

In urea the analysis is similar. The total number of groups titrated is 39. The total number of ionizable residues in α-chymotrypsin is 39 assuming that the guanidino groups may be titrated as well as the phenolic tyrosine groups. The guanidino group of arginine begins to titrate, in water, at *p*H 11.75 while in 8 M urea it begins to titrate at *p*H 12.0 (Fig. 4). Because of the favorable shift of pK_w in urea the titration may be continued to *p*H 13.5 so that more of the guanidino groups can be titrated.

DISCUSSION

The data obtained from the chymotrypsinogen titrations indicate that the titrimetric procedure quantitatively detects the ionizable residues present. The curves are reversible and would thus indicate that any appreciable irreversible denaturation does not involve large changes in the *pK* of the ionizing groups. No evidence for abnormally ionizing groups other than the 2 phenolic tyrosine groups was found. Chervenka (1961) mentions an abnormally low *p*H (1.7) for 3 carboxyl groups in chymotrypsinogen which ionized normally (*pK* 4) in urea-denatured chymotrypsinogen. The stoichiometry of the carboxylate region seems the same in urea or 0.15 M KCl. When identical samples of chymotrypsinogen are dissolved in 8 M urea–0.15 M KCl or in 0.15 M KCl, it can be assumed that the degree of protonation is identical even though the *p*H of these solutions is different. When sufficient acid is added to the 0.15 M KCl solution of chymotrypsinogen to protonate all the carboxyl groups, one would expect the same amount of acid to protonate the same number of groups in urea-denatured chymotrypsinogen. This is the case and argues against a shift to higher *pK* of any of the carboxylic groups in 8 M urea. Additional evidence that the carboxyl groups in chymotrypsinogen ionize normally can be obtained from the curves. The total proton binding reaches a maximum at *p*H 2.0. While this region of the curve is difficult to obtain with great accuracy, it is still possible to detect the occurrence of 3 groups with *pK* 1.7, since this would mean that 2 groups had not been titrated. This number of groups is well beyond the limit of error of the method. As a further check, samples of chymotrypsinogen were titrated to *p*H 1.5 and reversed. Exposure to this *p*H caused gelation at *p*H 4, but the portion of the curve from *p*H 2 to 4 was identical to that reported in Figure

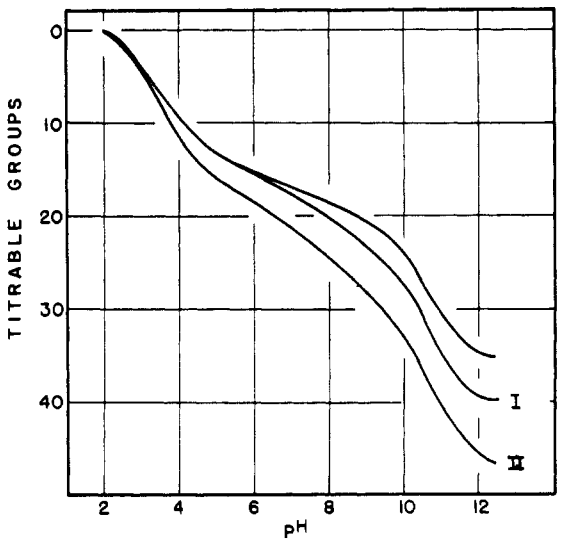

FIG. 6.—The correction for α-chymotrypsin autolysis during the titration in 0.15 M KCl at 20°. Curve I is the first titration. Curve II is the second titration after the first titration has been reversed rapidly to *p*H 2. The upper curve is the corrected ionization of α-chymotrypsin.

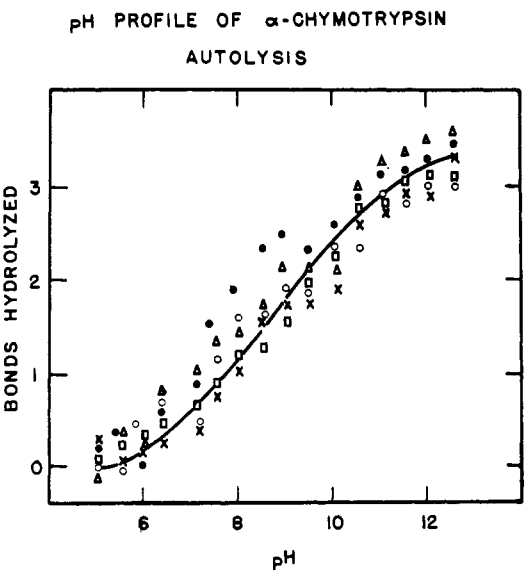

FIG. 7.—The *p*H profile of α-chymotrypsin autohydrolysis. The curves were obtained from the difference in titration curves obtained when α-chymotrypsin, 0.15 M KCl, 20°, was retitrated. Because various amounts of enzyme were used the autolysis rates were variable, so the total number of bonds hydrolyzed were arbitrarily adjusted to 3. Each symbol represents a different sample of α-chymotrypsin.

1, indicating that no additional groups were present below *p*H 2.0.

Chervenka maintained his solutions in 8 M urea at *p*H 3 and obtained the *pK* by extrapolation. It is possible that the discrepancy in the findings may be the result of exposure to *p*H 2.0 in the present study which may reversibly denature the protein. The denaturation of chymotrypsinogen at *p*H 2.0 is currently being investigated.

At the present time, we are unable to explain the apparent discrepancy between the theoretical isoelectric point and the observed value for chymotrypsinogen. We have assumed no ion-binding which is not, in fact, the case. Salt-free chymotrypsinogen in water has a *p*H 9.54 which is raised to *p*H 9.66 in 0.15 M KCl. This indicates chloride binding. If the isoelectric point is 9.66, at this point 19 groups are titrated. Since this represents the total number of cationic centers, the number of guanidino groups would be 3 rather than 4 as calculated. An alternative explanation would be to postulate that not all the groups below *p*H 9.66 are titrated. This, however, is not reasonable, since no group could be shown to titrate between *p*H 1.5 and 2.0. In addition, the total number of carboxylic acid groups (12) is identical in urea and KCl and agrees with the amino acid content. The curve of chymotrypsinogen obtained in 0.15 M KCl, analyzed independently of the isoelectric point, agrees with the amino acid content and the formol and urea titrations. This indicates that the observed isoelectric point is low for unexplained reasons.

α-Chymotrypsin also shows good agreement with the analytical data except in the extreme alkaline region. This portion of the curve is also difficult to obtain with great accuracy, especially since autolysis occurs and must be corrected. In a series of forty titrations on six different samples of α-chymotrypsin, the data indicates that 34.8 ± 0.3 groups are titrable. The failure of the curves to level out in the alkaline region indicates that more groups are titrating. Plotting the difference between the curves for chymotrypsinogen and the α-chymotrypsin will indicate the nature of the groups titrating in each *p*H region. These difference curves (Fig. 5) show that α-chymotrypsin has gained 2 groups with pK_{obs} 4.0, 2 groups with pK_{obs} 8.0, and one group titrating at *p*H 12.0. It is known (Rovery *et al.*, 1957) that chymotrypsinogen is activated to α-chymotrypsin by the removal of two dipeptides (containing 1 guanidino group) leaving an additional 2 free carboxyl groups and 2 α-amino groups. These can be expected to titrate as shown in Figure 5. The additional group may be a single guanidino group with *pK* 12.0 or a combination of the 3 guanidino groups with lower *pK*. These differences persist in 8 M urea (dashed curve, Fig. 5). The difference curves in urea indicate 2 groups with pK_{obs} 4.3 and 2 groups with pK_{obs} 7. An additional group titrates between *pK* 8 and 12. This portion of the difference curve is distorted because of the apparent differences in the ionization in 8 M urea of the tyrosines in α-chymotrypsin and chymotrypsinogen (Fig. 2). At *p*H 11.25 the additional groups appear to titrate indicating guanidino ionization.

The observed difference might be explained by α-chymotrypsin autolysis in 8 M urea. At the present time, reversal in 8 M urea is not quantitatively possible due to the dilution of the urea and the resultant shift in the *pK* of the ionizing groups. Autolysis in these solutions is not unlikely in view of the considerable quantities of enzyme used, but it is hardly likely that the autolysis would occur mainly at *p*H 11–12.

The finding of a lower *pK* for the guanidino groups in α-chymotrypsin as compared to chymotrypsinogen was not unexpected, since Erlanger (1960) had proposed such a shift and Herriott (1954) had shown a gain of one group in excess of that expected during chymotrypsinogen activation. The shift in *pK* is not nearly so great as that proposed by Erlanger (1960). The titration of guanidino groups in proteins is not unusual since they begin titrating at *p*H 12. The unusual protein is chymotrypsinogen, which shows no guanidino titration. Whether this is due to electrostatic or steric factors is unknown.

While no explanation for the guanidino shift is possible at this time, it is interesting to speculate on the importance of this finding on the catalytic activity of the enzyme. The hydrolysis of some substrates has been

shown (Bender *et al.*, 1962) to approach a maximum at *p*H 8.5 with no appearance of a normal bell-shaped profile. The *p*H dependence of α-chymotrypsin autolysis can be qualitatively obtained by subtracting the first titration curve from the second titration (Fig. 6). This gives the data shown in Figure 7. This curve indicates the amount of amino groups liberated as a function of time and *p*H since the titrations are performed from point to point at roughly 15-second intervals. The autolysis appears to increase with increasing *p*H. This could be explained by involving another center which dissociates at a higher *p*H. While this assumption is attractive in explaining the action of α-chymotrypsin against a protein substrate, considerably more quantitative data on the autolytic rate and the nature of the group involved must be obtained.

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Acetylcarboxypeptidase*

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Acetylation of carboxypeptidase with acetic anhydride increases esterase activity 6-fold and abolishes peptidase activity. These enzymatic changes are prevented by β-phenylpropionate and reversed by hydroxylamine. Acylation of the enzyme with other monocarboxylic acid anhydrides such as *i*-butyric, *n*-butyric, *n*-valeric, and propionic anhydrides also increases esterase and decreases peptidase activities. Though qualitatively the same the ensuing enzymatic changes are smaller than those observed with acetic anhydride. As in studies with acetylimidazole reported previously (Simpson *et al.*, 1963) the alterations in enzymatic activity correlate with the acetylation and deacetylation of two tyrosyl residues. Some kinetic and physical-chemical properties of Ac_A-carboxypeptidase are reported.

Previous studies from this laboratory have been concerned with the identification of those groups of the active site[1] of bovine pancreatic carboxypeptidase[2] which are involved in binding the catalytically essential zinc atom (Vallee *et al.*, 1960; Coleman and Vallee, 1961; Vallee *et al.*, 1961). Studies using site-specific reagents, the order and magnitude of the stability constants of a series of metallocarboxypeptidases, and complexometric titration data have indicated that the α-amino group of the N-terminal asparagine and the sulfhydryl group of the single cysteine residue of the enzyme constitute the metal binding site (Vallee *et al.*, 1960; Coleman and Vallee, 1961; Coombs and Omote, 1962).

While zinc is indispensable for activity, functional amino acid residues of the protein must also be essential in the catalytic process. Thus, peptide substrates of carboxypeptidase have been shown to form complexes with the metal-free apoenzyme (Coleman and Vallee, 1962a). Minimally, the aromatic or branched aliphatic side chain and the NH-function of the C-terminal amino acid as well as the NH-function of the penultimate amino acid are required for the formation of such a complex (Coleman and Vallee, 1962b). Clearly, these groups must interact with corresponding ones of the protein. In addition, the *p*H rate profile for peptidase activity suggests that a

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[1] The designation "active site" will refer specifically to the nitrogen-metal-sulfur bond essential for hydrolysis. "Active center" will refer to all those features of primary, secondary, and tertiary structure, *including* the "active site," which are required for substrate binding, specificity, or hydrolysis of the substrate.

[2] Carboxypeptidase will refer to carboxypeptidase A.