Specificity of Chymotrypsin B Toward Glucagon*

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Digests of glucagon with chymotrypsin α and chymotrypsin B have been compared using two-dimensional separation of the resulting peptides by high-voltage electrophoresis and paper chromatography as well as analysis of the amino-terminal amino acids by the fluorodinitrobenzene assay. Evidence is presented indicating that chymotrypsin B is less specific than chymotrypsin α. Under the reported conditions of digestion of glucagon, it was found that chymotrypsin B catalyzes the hydrolysis of peptide bonds involving the carboxyl group of leucine in addition to those split by chymotrypsin α. No evidence was found supporting a possible contamination of the enzyme preparation with a leucine esteroproteolytic enzyme.

Since the demonstration by Fruton (1948) that chymotrypsin B has a specificity toward synthetic substrates similar to chymotrypsin α, little work has been done on the enzymic properties of this enzyme. Keller *et al.* (1958) showed that the B enzyme hydrolyzed both *N*-acetyl-L-tyrosine ethyl ester and *N*-acetyl-L-tryptophan ethyl ester, the latter at a considerably slower rate. Rovery *et al.* (1961) corroborated their finding and in addition showed that the decrease in activity of chymotrypsin B toward the tryptophan ester in 30% methanol was much greater than for chymotrypsin α.

When consideration is taken of the wide application of proteolytic enzymes to studies of the primary structure of proteins, it was of interest to investigate the specificity of this enzyme toward a polypeptide chain and compare it to the well-established specificity of chymotrypsin α. Glucagon was chosen for this purpose since its primary sequence has been elucidated (Bromer *et al.*, 1957). In addition, it contains a variety of different amino acids and may be obtained commercially in a homogeneous form.[1]

EXPERIMENTAL

Glucagon Digestion.—Crystalline glucagon, reference standard (lot 258-234 B-167-1) and crystalline porcine glucagon[1] (lot 258-234 B-214 CP) were used during this investigation. The conditions of digestion were similar to those of Bromer *et al.* (1957), although in some experiments the ratio of enzyme to substrate was different. Chymotrypsin α, three times recrystallized, was purchased from Worthington Biochemical Corporation, Freehold, N. J. Chymotrypsinogen B was prepared and activated as previously described (Enenkel and Smillie, 1963). After activation, soybean trypsin inhibitor was added (weight ratio of enzyme to inhibitor, 1:5) in order to reduce the free tryptic activity to a minimum. Chymotrypsin B, free of trypsin, was prepared by chromatographing activated chymotrypsinogen B on carboxymethylcellulose columns under conditions identical to those described previously (Enenkel and Smillie, 1963). All digestions were carried out at *p*H 8.0 and 37 ± 0.2° in a *p*H-stat (Radiometer TTT1a titrator and Ole Dich recorder). The assembly was used with a temperature-controlled reaction vessel designed for small volumes (Dixon and Wade, 1958). In a typical experiment, 8.5 mg (2.44 μmoles) of glucagon in 10 ml of deionized water was introduced into the reaction vessel. The *p*H of the suspension was brought to 8.0, and 0.5 mg of enzyme (0.02 μmole) was added. The *p*H was kept at 8.0 by the automatic addition of 0.1 N NaOH (carbonate free). After 2 hours of digestion, a 5-ml aliquot was withdrawn from the digestion mixture, brought to *p*H 2.5 by the addition of 1 M HCl, heated in a boiling water bath for 3 minutes, and freeze-dried. To the remaining solution 0.25 mg of enzyme was added and the digestion was continued for a further 2 hours. The reaction was then stopped as above and the solution was again freeze-dried. In some experiments 8 hours of digestion were allowed, to simulate the conditions of Bromer *et al.* (1957).

Synthetic Substrates.—With the exception of benzoyl-L-leucine ethyl ester (BLEE)[2] all synthetic substrates were purchased from Mann Research Laboratories, Inc., New York. BLEE was prepared according to the method described by Ottesen and Spector (1960) for the preparation of benzoyl-L-tyrosine ethyl ester. Due to its limited solubility in water, it was dissolved in 95% ethanol (660 mg in 10 ml) and 100 μl of this solution was added to 2.4 ml of 0.01 M Tris buffer, *p*H 8.0, containing 0.02 M $CaCl_2$ and 0.1 M KCl for assays. ATEE, ATrEE, and L-phenylalanine ethyl ester were dissolved in the same buffer and assayed in the *p*H-stat as previously described (Enenkel and Smillie, 1963). The digestions of L-leucine ethyl ester, glycyl-L-leucyl-glycine, and tri-L-leucine could not be followed in the *p*H-stat. Solutions of the latter substrates (10 mM) were digested with the enzymes (molar ratio of enzyme to substrate, 1:500) at 23 ± 2° for 12 hours. Aliquots of the digests were examined by high-voltage electrophoresis at *p*H 2.2.

Identification of Digestion Products.—The products of digestion were isolated by a procedure essentially similar to the "fingerprint" technique of Katz *et al.* (1959). The completed "fingerprints" were sprayed lightly with ninhydrin solution (0.05% ninhydrin in 2 N acetic acid—absolute ethanol, 1:3; Matheson *et al.*, 1961). The colors were allowed to develop overnight at room temperature. Some peptides containing tryptophan and valine did not stain when sub-

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[1] We are indebted to Eli Lilly and Co. for supplies of this material.

[2] The following abbreviations have been used: BLEE, benzoyl-L-leucine ethyl ester; Tris, tris(hydroxymethyl)-aminomethane; ATEE, *N*-acetyl-L-tyrosine ethyl ester; ATrEE, *N*-acetyl-L-tryptophan ethyl ester; DNP-, dinitrophenyl-; DFP, diisopropyl fluorophosphate.

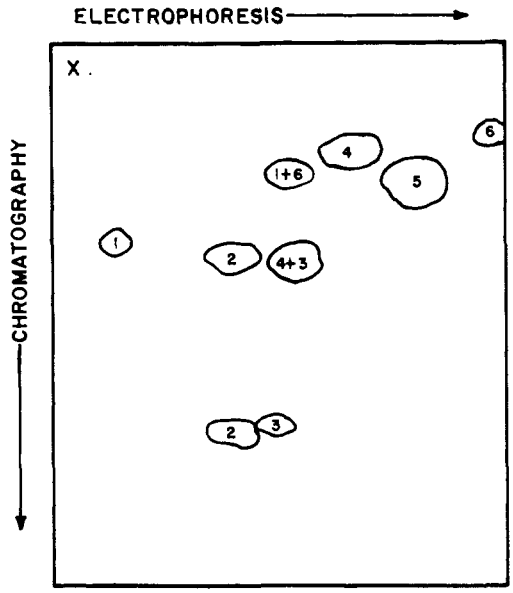

FIG. 1.—Peptide map of chymotrypsin α digest of glucagon.

jected to the above treatment. They could be detected under ultraviolet light. All spots were cut out and eluted with 5–10 ml of demineralized water. The samples were dried *in vacuo*, taken up in 0.75 ml of constant boiling HCl, and hydrolyzed in sealed evacuated tubes for 16 hours at 110 ± 2°. After removal of the hydrochloric acid *in vacuo*, the residue was dissolved in a minimum amount of deionized water and subjected to two-dimensional high-voltage electrophoresis and chromatography according to the method of Richmond and Hartley (1959). Standard amino acid mixtures (0.1 μmole of each amino acid) were always subjected to hydrolysis, electrophoresis, and chromatography along with the peptides to allow accurate identification and estimation (± 15%).

Amino-Terminal Analysis.—The reaction of peptide mixtures with fluorodinitrobenzene was conducted according to Sanger and Thompson (1953). All the operations with DNP-amino acids were conducted in the dark to minimize their destruction. Standard mixtures of DNP-amino acids (Mann Research Laboratories, Inc) were subjected to the same treatment to allow the estimation of recoveries. The ether-soluble DNP-amino acids were separated by the *tert*-amyl alcohol–phthalate system of Blackburn and Lowther (1951). The water-soluble DNP-amino acids were subjected to descending chromatography in the butanol–acetic acid–water (4:1:5) system of Levy and Chung (1953).

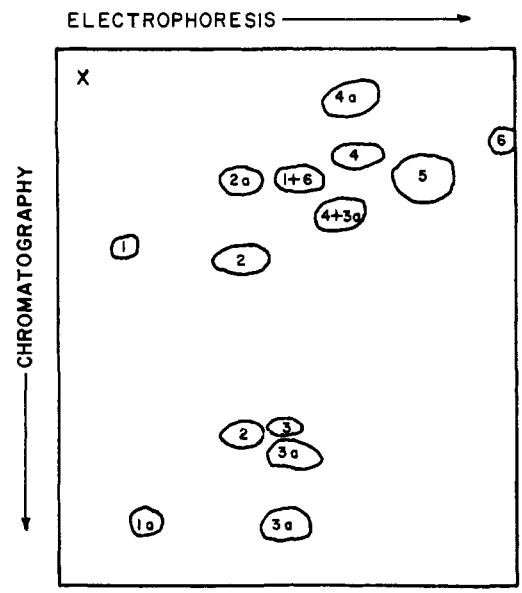

FIG. 2.—Peptide map of chromatographed chymotrypsin B (trypsin free) digest of glucagon.

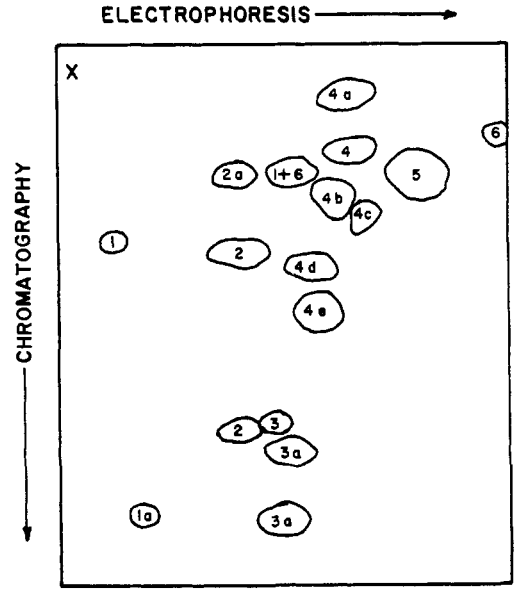

FIG. 3.—Peptide map of activated chymotrypsinogen B digest of glucagon (soybean trypsin inhibitor added to inhibit trypsin).

RESULTS

The peptide maps resulting from the digestion of glucagon with chymotrypsin α, chromatographed chymotrypsin B, and activated chymotrypsinogen B are shown in Figures 1, 2, and 3. Each circle represents a peptide, and the numbers in the circles refer to Table I, which lists the amino acid sequence of each

TABLE I

AMINO ACID SEQUENCES OF PEPTIDES LIBERATED FROM GLUCAGON BY CHYMOTRYPSIN α AND B

Peptide No.[a]	Amino Acid Sequence
1	thr·ser·asp·tyr
1a	leu
2	leu·met·aspNH_2·thr
2a	met·aspNH_2·thr
3	val·gluNH_2·try
3a	val·gluNH_2·try·leu
4	leu·asp·ser·arg·arg·ala·gluNH_2·asp·phe
4a	asp·ser·arg·arg·ala·gluNH_2·asp·phe
4b	leu·asp·ser·arg
4c	arg·ala·gluNH_2·asp·phe
4d	arg·ala·gluNH_2·asp·phe·val·gluNH_2·try
4e	arg·ala·gluNH_2·asp·phe·val·gluNH_2·try·leu
5	his·ser·gluNH_2·gly·thr·phe
6	ser·lys·tyr
1 + 6	thr·ser·asp·tyr·ser·lys·tyr
4 + 3	leu·asp·ser·arg·arg·ala·gluNH_2·asp·phe·val·glu(NH_2)·try
4 + 3a	leu·asp·ser·arg·arg·ala·gluNH_2·asp·phe·val·gluNH_2·try·leu

[a] See Figs. 1, 2, and 3.

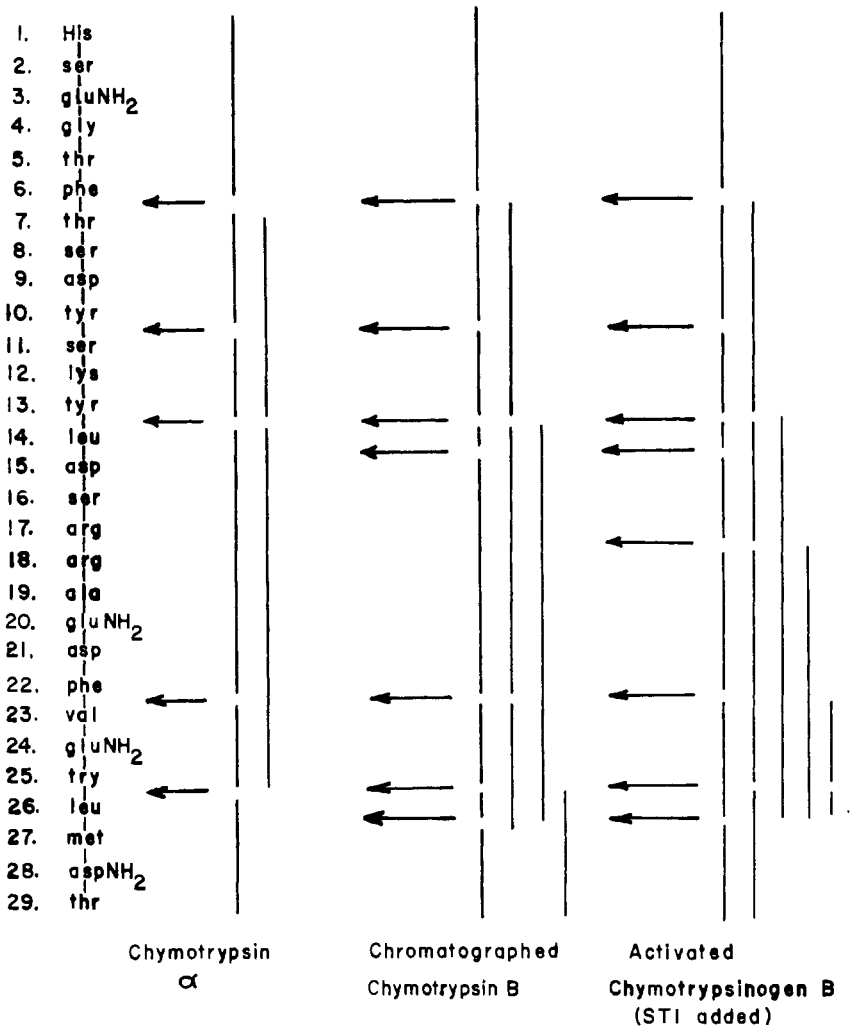

FIG. 4.—The action of chymotrypsin α, chromatographed chymotrypsin B, and activated chymotrypsinogen B (STI [soybean trypsin inhibitor] added) on glucagon.

peptide. In cases where a peptide bond was only partially hydrolyzed, peptides were produced corresponding to a larger fragment of the glucagon molecule. These peptides are designated by a composite number to indicate that their total amino acid composition corresponds to the sum of two smaller peptides found on the map. Figure 4 relates the findings of the peptide maps to the primary structure of glucagon.

It will be noted that at least two spots on each peptide map bear identical numbers. For example, both spots numbered 2 were found to contain the amino acids leucine, methionine, aspartic acid, and threonine. It is believed that partial deamidation of the asparagine residue present in glucagon at this position is responsible for the encountered observation. By the same token, the circles numbered 3a probably result from the partial deamidation of a glutamine residue. When digests of glucagon with chymotrypsin B were first examined, the peptide val·gluNH$_2$·try (peptide 3) could not be found as a ninhydrin-positive spot. However, because of the presence of tryptophan, ultraviolet light proved to be more efficient. A re-examination of some sprayed papers after 2 or 3 weeks revealed that very faint blue spots had developed where ultraviolet light had demonstrated the presence of these peptides (3 and 3a). These findings can probably be explained by observations made in other laboratories, where it was found that peptides containing valine gave low color yields with ninhydrin (Bromer *et al.*, 1957; Craig *et al.*, 1950; Harfenist, 1953).

Base consumption as a function of time during the hydrolysis of glucagon with chymotrypsin α and chymotrypsin B gave the first indication of lesser degree of specificity of the latter enzyme (Fig. 5). A comparison of the total uptake of NaOH during a period of digestion of 4 hours demonstrates that chymotrypsin B has hydrolyzed a larger number of available peptide bonds than chymotrypsin α. Assuming a pK of 7.3 for the amino groups (Murachi and Neurath, 1960), it can be calculated from the base uptake during the first 2 hours of digestion that chymotrypsin α and chymotrypsin B have cleaved the equivalent of 5.7 and 7.4 peptide bonds, respectively. Two other interesting features are apparent from this figure. First, a very fast reaction during the first 5 minutes of digestion is followed by a much slower uptake of NaOH. Second, when an additional aliquot of the enzyme is added to the digestion mixture 2 hours after the start of the reaction, only a small and rapid uptake of NaOH can be observed, followed by a slower rate of reaction than that immediately before the second addition of the enzyme. The peptide map of Figure 1 (digest of glucagon with chymotrypsin α) essentially corroborates the results of Bromer *et al.* (1957). The only difference between the two sets of data is found in the presence of peptides 4 + 3 and 1 + 6, which

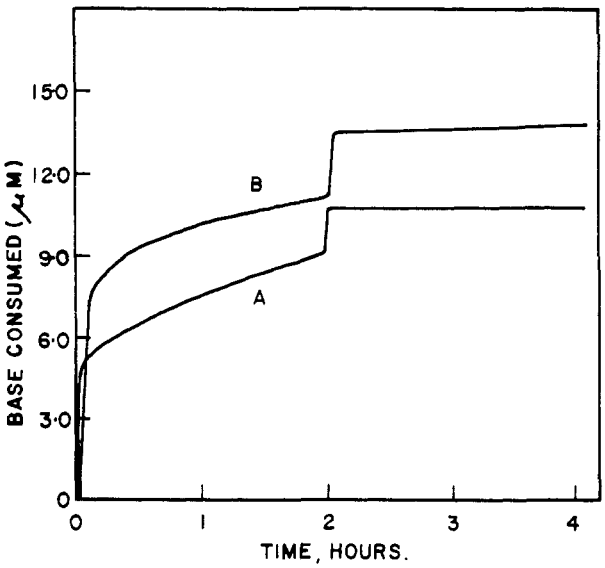

FIG. 5.—Rate of hydrolysis of glucagon (2.44 μmoles) by chymotrypsin α (curve A), chymotrypsin B (curve B); molar ratio of enzyme-glucagon, 1:120; at 2 hours fresh enzyme was added to give final ratio 1:60.

results from the incomplete cleavage of one tyrosine and one phenylalanine bond. Even 8 hours of digestion did not change the results significantly. However, in the work of Bromer *et al.* it is possible that strong adsorption to the Dowex-X2 resin may have prevented the detection of these larger fragments. Apart from this discrepancy, the results agree fully with the well-established specificity of chymotrypsin α.

The peptide map of Figure 2 and the data of Table I demonstrate that chymotrysin B not only cleaves those bonds split by chymotrypsin α but in addition catalyzes the hydrolysis of peptide bonds adjacent to leucine residues in glucagon. This becomes evident from the appearance of five peptides not present in digests of glucagon with chymotrypsin α. Further evidence for these splits arose during the early stages of this study when it was observed that trypsin used for the activation was not completely inhibited by added soybean trypsin inhibitor and caused the cleavage of the arg-arg bond of glucagon (positions 17 and 18). Figure 3 shows a representative peptide map of such a digest. Identification of the amino acids of each peptide made possible only one interpretation of the results. Tryptic digestion causes the cleavage of the arg-arg bond of peptides 4, 4a, and 4 + 3a. These results gain more weight in the light of the findings of Bromer *et al.* (1957) which demonstrate that this particular bond in glucagon is highly susceptible to tryptic hydrolysis. It should also be mentioned that chymotrypsin B was found to catalyze an even larger number of peptide bonds when incubated with glucagon under different conditions (0.2% glucagon, ratio of enzyme to substrate 1:50, 37°, 4 hours). Although no detailed studies of representative peptide maps have been undertaken, some available evidence indicates that bonds adjacent to aspartic acid are also cleaved.

From the above data, it is impossible to make definitive statements about the rates at which different peptide bonds are hydrolyzed. However, results from digests terminated after 5 minutes of hydrolysis (end of rapid reaction) indicate that both enzymes attack one phenylalanine bond (position 6 along the peptide chain) and one tyrosine bond (position 13) more rapidly than any others. These findings are in accord with the results obtained from 4-hour digests of glucagon with chymotrypsin B. Figure 4 indicates that only the bonds adjacent to amino acid residues 6 and 13 are hydrolyzed to the extent of 100%. Chymotrypsin α, in addition, cleaves the peptide bond between residues 25 and 26 (involving the carbonyl group of tryptophan) completely.

TABLE II

DNP-AMINO ACIDS ISOLATED FROM DIGESTS OF GLUCAGON WITH CHYMOTRYPSIN α AND CHROMATOGRAPHED CHYMOTRYPSIN B[a]

DNP-amino Acid	Chymotrypsin α Digestion 2 Hours	Chymotrypsin α Digestion 4 Hours	Chymotrypsin B Digestion 2 Hours	Chymotrypsin B Digestion 4 Hours
Aspartic acid	0.01	0.01	0.25	0.31
Methionine	—	—	0.36	0.45
Valine	0.49	0.56	0.32	0.38
Threonine	0.68	0.75	0.59	0.68
Serine	0.18	0.20	0.19	0.23
Leucine	1.25	1.40	0.73	0.79

[a] Moles per mole of glucagon.

One possible way of confirming the above results was to react aliquots of the digests with fluorodinitrobenzene and separate the formed DNP-amino acids after hydrolysis. Results from such studies should also give a reasonably quantitative picture of the peptides present in the digests. Table II shows the findings from 2- and 4-hour digests of glucagon with chymotrypsin α and chymotrypsin B. The liberation of 0.01 μmole of aspartc acid from digests with chymotrypsin α does not seem significant and may arise from an impurity of the enzyme itself. Together with the absence of methionine, it demonstrates unequivocally that chymotrypsin α attacks peptide bonds in glucagon adjacent to aromatic amino acid residues only. The findings with respect to chymotrypsin B, however, can be explained only on the basis of hydrolysis of two leucine peptide bonds in addition to those involving aromatic residues. It is also interesting to note that no significant increase in the quantities of amino-terminal amino acids is found after 4 hours, although neither enzyme has achieved the complete hydrolysis of all susceptible peptide bonds of glucagon after 2 hours of digestion. Denaturation of the enzyme as a possible explanation for this phenomenon can be ruled out, because an additional aliquot of chymotrypsin α or chymotrypsin B was added after 2 hours.

The data on the hydrolysis of some synthetic amino acid esters and peptides by the two enzymes are presented in Table III. It is obvious that no essential

TABLE III

HYDROLYSIS OF AMINO ACID ESTERS AND PEPTIDES BY CHYMOTRYPSIN α AND B

Substrate	Chymotrypsin α (%)	Chymotrypsin B (%)
ATEE[a]	100	100
ATrEE	37	22
L-Phenylalanine ethyl ester	6	2
BLEE	1.6	1.2
L-Leucine ethyl ester	Complete[b]	Complete[b]
tri-L-Leucine	None[b]	None[b]
Gly-leu-gly	None[b]	None[b]

[a] The value of k' for hydrolysis of ATEE represents 100%. [b] Time of hydrolysis, 12 hours.

difference between the two enzymes is found with regard to the substrates tested. Both catalyze the hydrolysis of leucine esters and neither has any demonstrable activity against such peptides as L-trileucine or glycyl-L-leucyl-glycine.

Absence of Leucine Esteroprotease Contamination.— Since it was clear from these data that our preparations of chymotrypsin B catalyzed the hydrolysis of peptide bonds involving the carboxyl group of leucine, it was of some importance to establish that this leucine esteroproteolytic activity was an inherent component of the chymotrypsin molecule. Toward this end, the following series of experiments was carried out.

(1) Two samples of chymotrypsinogen B were dissolved in 0.1 M Tris buffer, *p*H 8.0. Aliquots withdrawn from these samples showed 0.02% of free activity against ATEE and no detectable activity against BLEE. After 10 and 30 minutes of activation, the k' for ATEE was 2.98 and 2.95, respectively, and for BLEE was 0.034 and 0.034, respectively.

(2) A 1% solution of chymotrypsin B in 0.1 M Tris buffer, *p*H 8.0, was incubated with 0.0001 M DFP. Samples were removed after 5, 10, 20, and 60 minutes of incubation and tested against ATEE and BLEE. Activities toward both substrates were 30, 12, 1.5, and 0.5% of the zero-time values, respectively.

(3) During the preparative procedures for chymotrypsinogen B (Enenkel and Smillie, 1963), samples were tested for their potential activity toward both ATEE and BLEE. The following results were obtained; the figures in brackets represent the ratios of activities toward BLEE and ATEE; protein powder 1 (1.17×10^{-2}); protein powder 3 (1.14×10^{-2}); and three preparations of chymotrypsinogen B after CM-cellulose chromatography (1.20, 1.12, and 1.10 $\times 10^{-2}$).

(4) The ratios of k' for ATrEE and BLEE were evaluated for seven different fractions of a chromatographic peak of chymotrypsinogen B eluted from CM-cellulose by a citrate gradient (Enenkel and Smillie, 1963). Table IV lists these ratios together with the number of each fraction and the absorbancy at 280 mμ. Although the agreement between the ratios cannot be considered perfect, no trend can be detected.

(5) Chymotrypsin B was assayed against BLEE and ATEE in the presence of 0.005 M indole, a competitive inhibitor of chymotrypsin (Huang and Niemann, 1953). The enyzme was inhibited to the extent of 80% and 50%, respectively.

Discussion

During the studies of the amino acid sequence of glucagon, Bromer *et al.* (1957) demonstrated that chymotrypsin α catalyzed the hydrolysis of five peptide bonds adjacent to aromatic amino acid residues (2 tyrosine, 2-phenylalanine, and 1 tryptophan residue) along the polypeptide chain. The separation of six peptides was achieved on Dowex-X2 resin and each peptide was characterized with regard to its amino acid composition and sequence. It was further shown that no two peptides formed overlapping sequences, which meant that the proteolysis of glucagon by chymotrypsin α was essentially complete after 8 hours of hydrolysis (80–90% as revealed by amino-terminal analyses of digests). Additional studies on digests of the hormone with trypsin and subtilisin enabled these investigators to propose the amino acid sequence of glucagon. It was therefore possible for the present authors to determine the bond(s) hydrolyzed by chymotrypsin B from amino acid analyses of the peptides separated by the two-dimensional system. The results presented in this paper are in essential agreement with those reported by Bromer *et al.* (1957). Only the presence of the peptides 4 + 3 and 1 + 6 in our digests was unexpected and may be explained by the different technique for the separation of the peptides used in this laboratory.

Table IV

Ratios of Potential Activities of Chymotrypsinogen B toward ATrEE and BLEE over a Column Peak on CM-cellulose

Fraction Number	Absorbancy (280 mμ)	k'_{ATrEE}/k'_{BLEE}
1	0.128	17
2	0.685	20
3	1.030	19
4	1.000	20
5	0.642	20
6	0.345	21
7	0.319	23

In the digestion of glucagon with chymotrypsin B, it is clear from the above results that in addition to the peptide bonds of tyrosine, phenylalanine, and tryptophan, the two bonds involving the carboxyl groups of leucine were cleaved. It was of some importance to ascertain whether the leucine esteroproteolytic activity was an inherent property of the enzyme or was due to a contaminating enzyme. The following lines of evidence support the former view. Leucine esterase activity requires activation with trypsin since the zymogen shows no activity against BLEE. Diisopropylfluorophosphate inhibits leucine esterase activity at the same rate as it does tyrosine esterase activity. The ratios of activities toward BLEE and ATEE remain constant throughout the purification stages of the enzyme. The ratios of activities remain essentially constant over a chromatographic peak on CM-cellulose. Indole, a competitive inhibitor of chymotrypsin, reduces the rates of hydrolysis of both ATEE and BLEE. Chymotrypsin B, purified by gradient CM-cellulose chromatography, still hydrolyzes the leucine bonds of glucagon. If the leucine esterase activity were not an intrinsic component of the chymotrypsin B activity, then the inactive and activated leucine esterase would have to behave identically to chymotrypsinogen B and chymotrypsin B, respectively, during column chromatography. The weight of evidence favors the conclusion that the leucine esteroprotease activity is an inherent component of the chymotrypsin B activity.

Although a lesser degree of specificity of chymotrypsin B than chymotrypsin α toward the peptide bonds of glucagon is well substantiated by the results presented above, no significant differences in the rates of the hydrolysis of synthetic substrates by the two enzymes could be demonstrated. In a recent communication, Konigsberg and Hill (1962) have observed that either chymotrypsin α or pepsin can hydolyze particular peptide linkages at much faster rates than other potentially sensitive residues. As pointed out by these authors and by Hirs *et al.* (1960), the differential rates of hydrolysis may be governed by a longer sequence of the polypeptide chain or by the conformation of the peptide itself. There is sufficient evidence from the present study that these suggestions apply equally well to chymotrypsin B. Studies on the uptake of NaOH as a function of time together with data from amino-terminal analyses, indicate clearly that the hydrolysis of certain peptide bonds does not reach completion even though they appear to be rapidly cleaved in the early stages of the digestion. Prolonged

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.