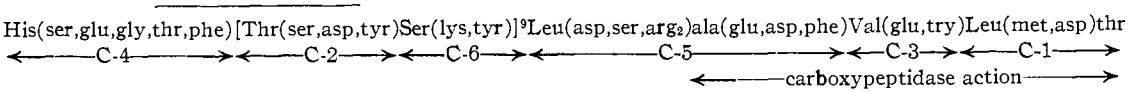
in turn is next to the C-terminal peptide. No clues are as yet available for the location of peptides C-2 and C-6, although they are obviously within the protein chain and must be adjacent to each other. Additional information is needed to determine which is adjacent to peptide C-4 and which to peptide C-5.

(9) Brackets indicate that the sequence of peptides C-2 and C-6 has not yet been determined.

The evidence available to this point may be summarized in the following partial structure of glucagon



Acknowledgment.—The authors wish to acknowledge the expert technical assistance of W. A. Tandy and R. G. Scheib, and the paper chromatographic separations performed by H. L. Bird and C. T. Pugh.

INDIANAPOLIS 6, INDIANA

[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

The Amino Acid Sequence of Glucagon. III. The Hydrolysis of Glucagon by Trypsin

BY W. W. BROMER, A. STAUB, L. G. SINN AND OTTO K. BEHRENS

RECEIVED NOVEMBER 19, 1956

Tryptic digestion of glucagon caused rapid hydrolysis of two peptide bonds and slower splitting of three additional bonds. The peptide fragments resulting from both a 2.25 and a 50 hr. digestion were isolated by use of resin chromatography and electrophoresis. The unique composition of the peptide fragments permitted the correlation of existing evidence into the following partial amino acid sequence of glucagon: His(ser,glu,gly,thr,phe)thr(ser,asp,tyr)ser.lys.tyr(leu,asp,ser,arg)-arg.ala(glu,asp,phe)val(glu,try)leu(met,asp)thr.

Introduction

The first two papers of this series[1,2] have described the amino acid composition of glucagon, the end group analysis and the characterization of products of degradation by carboxypeptidase and chymotrypsin. Relative positions within the glucagon chain were assigned to 4 of the 6 peptides isolated from the chymotryptic digestion. To provide additional information useful for structural analysis glucagon has been hydrolyzed with another proteolytic enzyme, trypsin. The products of both 2.25 and 50 hr. tryptic hydrolyses of glucagon have been isolated and characterized.

Experimental

Materials.—Crystalline glucagon, lot 208-158B-292A, was used throughout this enzymatic study. Twice-recrystallized trypsin, lot T320, was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. The chymotrypsin content of this trypsin preparation was less than 0.2% according to analyses by the method of Ravin, *et al.*,[3] using N-benzoyl-DL-phenylalanine β-naphthyl ester as the substrate.

Hydrolysis of Glucagon by Trypsin.—An 0.085% solution of glucagon was incubated with 0.005% trypsin in a 0.05 *M* sodium citrate solution containing 1 mg. per ml. of $CaCl_2$. The enzyme:substrate mole ratio was approximately 1:100. The digestion was performed at 25°, *p*H 7.8, for as long as 50 hr. Excessive stirring of the incubate was avoided since trypsin was found to be inactivated under this condition. Aliquots were removed at various intervals for dinitrophenylation and for determination of the rate of appearance of the N-terminal residues of newly-formed peptides. Preliminary studies of the rate of hydrolysis provided evidence that certain bonds were split very rapidly, while others were ruptured quite slowly. Hence, in the preparative experiments aliquots were removed at both 2.25 and 50 hr.; these solutions were adjusted to *p*H 3 with HCl, were heated to 90° for 2 min., and were lyophilized.

Fractionation of the Trypsin Hydrolyzate.—The lyophilized digests were not completely soluble in the sodium citrate buffer (*p*H 3.0, 0.1 *N*) used in the Dowex 50-X2 chromatographic separation. The precipitate in each digest was peptide in nature, somewhat soluble in dilute alkali, but insoluble in the *p*H range 2.5 to 6.5. Because of the insolubility in the *p*H range of the buffers used in the Dowex 50 chromatography, the precipitate was removed by centrifugation, washed with cold dilute acid, redissolved in dilute alkali (*p*H 8.5 to 9.5) and frozen. The combined washes and supernatant were subjected directly to Dowex 50-X2 chromatography on 1.5 × 50 cm. columns as described in a previous report.[2]

End group analysis[4] of the acid-insoluble materials showed that the 2.25 hr. precipitate was not homogeneous, but the 50 hr. precipitate, isolated in small quantity, appeared to represent a single component. Because of the relatively high purity and small yield of the 50 hr. precipitate no further fractionation was attempted. However, the material which precipitated from the 2.25 hr. digest was subjected to electrophoresis on both starch and paper in 0.1 *N* sodium acetate (*p*H about 7.2) at a milliamperage and voltage of 15 and 300, respectively. The peptide material was eluted from the paper with 1% NH_4OH, and from one-half or one cm. sections of the starch with 5 ml. of water. The peptide material was located in both cases by use of the ninhydrin reagent.[5]

Results

Rate of Hydrolysis.—Glucagon was previously shown to contain one lysine and two arginine residues.[1] Because of the well-accepted specificity behavior of trypsin[6-10] in splitting only those bonds involving the carboxyl groups of these residues, it was expected that 3 bonds in glucagon would be split and 4 hydrolytic products would be formed. The rate of hydrolysis data presented in Fig. 1 show clearly that the tryptic hydrolysis of glucagon did

(1) Reference to Paper I in this series, THIS JOURNAL, **79**, 2794 (1957).
(2) Reference to Paper II in this series, *ibid.*, **79**, 2798 (1957).
(3) H. A. Ravin, P. Bernstein and A. M. Seligman, *J. Biol. Chem.*, **208**, 1 (1954).
(4) A. L. Levy, *Nature*, **174**, 126 (1954).
(5) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).
(6) C. H. W. Hirs, S. Moore and W. H. Stein, *ibid.*, **219**, 623 (1956).
(7) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951).
(8) F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 353, 366 (1953).
(9) P. H. Bell, THIS JOURNAL, **76**, 5565 (1954).
(10) W. F. White and W. A. Landmann, *ibid.*, **77**, 1711 (1955).

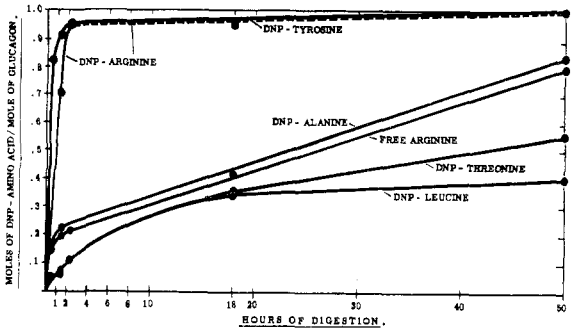


Fig. 1.—A study of the rate of the tryptic hydrolysis of the peptide bonds of glucagon, as determined by the dinitrophenylation method. An 0.085% solution of glucagon was digested with trypsin (0.005%) at *p*H 7.8 and 25°. Free arginine was determined by chromatography on Dowex 50-X8 resin.

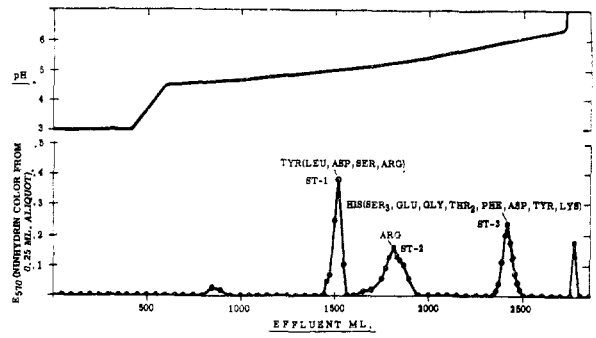


Fig. 2.—Separation of the acid-soluble peptides resulting from the 2.25 hr. tryptic hydrolysis of 146 mg. of glucagon. Chromatography was performed on a 1.5 × 50 cm. column of Dowex 50-X2 resin. Five-ml. effluent fractions were collected. A 0.2 *N* NaOH solution was used to clean the resin column, producing the final sharp rise in *p*H of the effluent.

not proceed in the expected manner. Within 2 hr. nearly complete hydrolysis of 2 peptide bonds occurred, since both di-DNP-tyrosine and DNP-arginine were obtained in high yield. With longer incubation, increasing quantities of DNP-alanine, DNP-threonine, DNP-leucine and free arginine were observed. None of these amino acids appeared to be quantitatively released even after 50 hr. digestion. The free arginine was determined by the analysis of aliquots of the digest on Dowex 50 columns according to the method of Moore and Stein.[11]

It is apparent that two peptide bonds were hydrolyzed rapidly during the trypsin digestion. At least some of these primary peptides must have been further degraded to yield 4 or more additional components.

Peptides from the 2.25 Hour Tryptic Digestion.—The effluent curve presented in Fig. 2 was obtained from the Dowex 50-X2 chromatography of the supernatant from 146 mg. (41.7 μmoles) of glucagon after digestion with trypsin for 2.25 hr. Separation of the components of the precipitate by paper electrophoresis is represented in Fig. 3 by the photograph of a paper strip developed with ninhydrin reagent. The quantitative amino acid analyses and recoveries of all the hydrolytic fragments are summarized in Table I.

TABLE I

PEPTIDES FROM THE 2.25 HOUR TRYPTIC HYDROLYSIS OF GLUCAGON

Peptide number	Peptide compn. and anal.[a]	Yield, %
ST-1	tyr(leu,asp,ser,arg)	
	moles 1.0 1.0 1.0 1.0 1.0	70
ST-2	arg	20
ST-3	his(ser$_2$,glu,gly,thr$_2$,phe,asp,tyr,lys)	
	moles 0.8 3.2 1.2 1.0 2.1 1.0 1.0 1.0 1.1	30
ST-PA	arg(ala,glu$_2$,asp$_2$,phe,val,try,[b]leu,met,thr)	
	moles 1.0 1.0 2.0 1.8 0.9 1.1 1.0 0.9 0.7 1.2	~50
ST-PB	ala(glu$_2$,asp$_2$,phe,val,try,[b]leu,met,thr)	
	moles 1.0 2.1 2.1 0.7 1.0 1.0 1.3 0.5 1.3	~25

[a] All peptides analyzed by Moore and Stein method.[11] Small amounts of peptides ST-PA and ST-PB available for analysis. [b] Chemical analysis.[12]

DNP-end group analysis of the acid-insoluble material provided evidence for two major components, one containing alanine as the N-terminus and the other arginine as the end group. No other DNP-amino acids were observed. Traces of ninhydrin-positive material other than peptides ST-PA and ST-PB were found by paper and starch electrophoresis; however, the quantity of material was not sufficient for identification. The yields of fragments ST-PA and ST-PB were estimated on the basis of amino acid and end group analyses prior to electrophoretic separation. The amino acid analyses were performed on small quantities of peptides isolated after electrophoretic separation.

The three fragments represented by the effluent curve in Fig. 2 were isolated, desalted and analyzed as described in a previous report.[2] No other peptide material was detected. DNP-end group and quantitative amino acid analyses, along with the Dowex 50 data, demonstrated the high purity of the fragments. The yield of arginine (ST-2) was calculated from the direct analysis of aliquots of the reaction mixture according to the method of Moore and Stein[11] for basic amino acids. Chromatography before and after hydrolysis provided additional evidence for free arginine.

These 5 isolated fragments may be considered in light of the splits predictable from the data on the rate of hydrolysis. Three major degradation products were anticipated after 2.25 hr. digestion. These products should contain histidine, tyrosine and arginine as N-terminal residues (*cf.* Fig. 1). The data in Table I indicate that those three peptides isolated in the highest yield, ST-1, ST-3 and ST-PA, contain these amino end groups. Furthermore, the sum of the amino acids in these three degradation products is identical to the amino acid composition of the entire glucagon molecule. The relatively low yield of ST-3 is perhaps understandable when the large size and amino acid composition of the peptide are considered. The presence of two basic residues and two aromatic residues probably tended to retard elution of the peptide from the Dowex 50 column and from the resin during the desalting step.

In addition to the two major splits during the first 2.25 hours, the rate study data indicated that

(11) S. Moore and W. H. Stein, THIS JOURNAL, **192**, 663 (1951).
(12) J. R. Spies and D. C. Chambers, *Anal. Chem.*, **20**, 30 (1948).

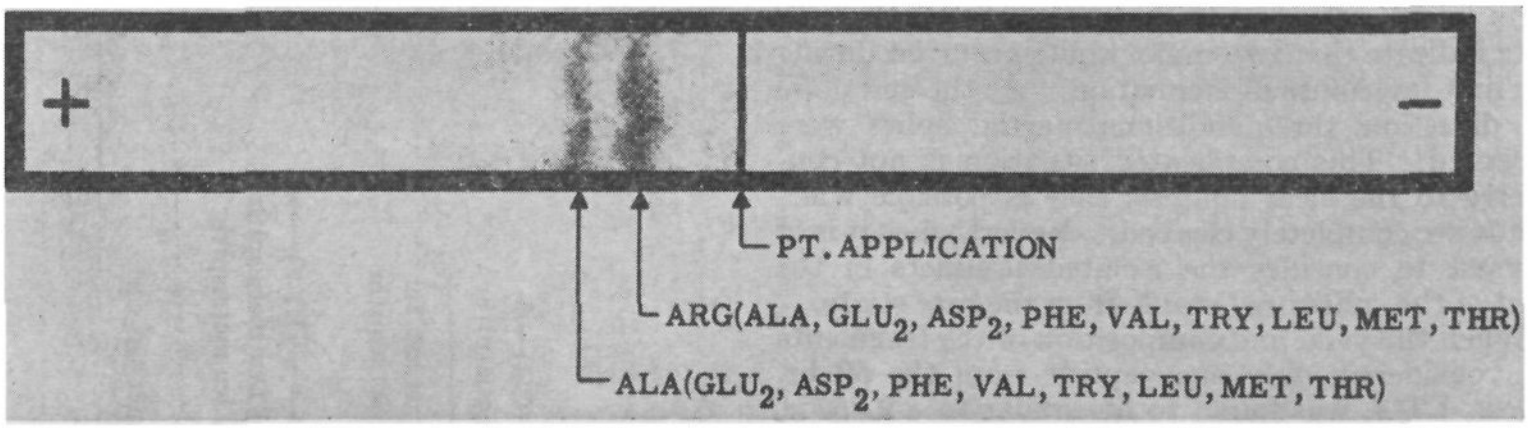


Fig. 3.—Photograph of the paper electrophoretic separation of the components of the acid-insoluble material from the 2.25 hr. tryptic digestion of glucagon. Electrophoresis was performed for 4 hr. at 5° in 0.1 *N* sodium acetate solution (*p*H 7.2), with a milliamperage and voltage of 15 and 300, respectively.

lesser splits had occurred which involved free arginine and N-terminal alanine. The two remaining fragments which were isolated, ST-2 and ST-PB, are in complete agreement with this prediction. On the basis of the yields and the composition of the fragments, it is probable that ST-2 and ST-PB arose from the degradation of ST-PA. The tryptic liberation of N-terminal arginine is somewhat unexpected in view of the resistance to tryptic hydrolysis of amino-terminal lysine in ribonuclease, as found by Hirs, Moore and Stein.[6]

Small yields (10%) of DNP-leucine and DNP-threonine were also detected in the rate study; no corresponding peptides were detected, possibly because the quantities were too small.

Peptides from the 50 Hour Tryptic Digestion.—Separation of the acid-soluble degradation products from 165.2 mg. (47.2 μmoles) of glucagon formed by a 50 hr. tryptic digestion is represented by the Dowex 50-X2 effluent curve in Fig. 4. Peak LT-5

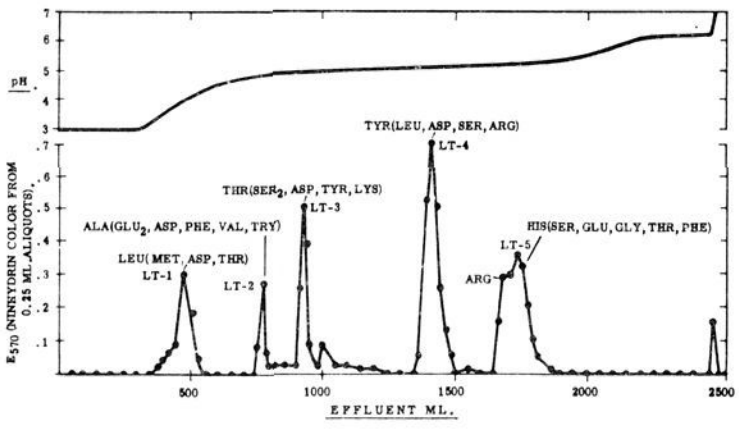


Fig. 4.—Separation of the acid-soluble peptides resulting from the 50 hr. tryptic hydrolysis of 165.2 mg. of glucagon. Chromatography was performed on a 1.5 × 50 cm. column of Dowex 50-X2 resin. Five-ml. effluent fractions were collected. A 0.2 *N* NaOH solution was used to clean the resin column, producing the final sharp rise in *p*H of the effluent.

was found by end group analysis to represent two components, one with arginine as the N-terminus and the other containing N-terminal histidine. Rechromatography of this mixture on Dowex 50-X8 columns described by Moore and Stein[11] for basic amino acids resulted in the separation of the two components, as demonstrated by the elution curve in Fig. 5. The elution pattern of peak LT-5B indicated that this component was identical with free arginine. Rechromatography after acid hydrolysis provided further evidence that a single arginine residue had been liberated. The analysis and recovery of the various isolated fragments is presented in Table II. End group and quantitative amino acid analysis provided evidence

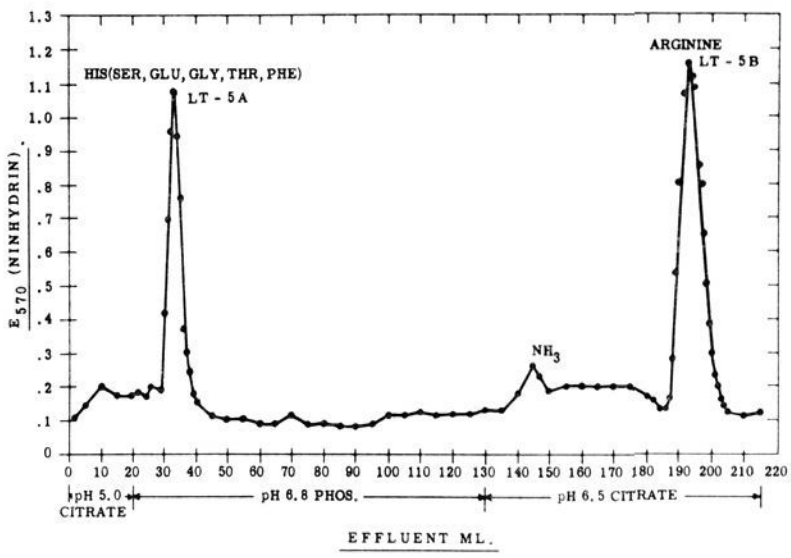


Fig. 5.—Rechromatography of the material represented by peak LT-5. Separation into two components was accomplished on a 0.9 × 15 cm. column of Dowex 50-X8 resin.

that LT-1 represented more than 80% of a single component. No evidence of amino acid or peptide impurities in the other peptides was observed, except that the acid-insoluble material contained a small amount of peptide probably identical to ST-PA.

TABLE II

PEPTIDES FROM THE 50 HOUR TRYPTIC DIGESTION OF GLUCAGON

Peptide no.	Peptide compn. and anal.[a]	Yield, %
LT-1	leu(met,asp,thr)	
	moles 1.0 0.7 1.0 1.0	35
LT-2	ala(glu$_2$,asp,phe,val,try[b])	
	moles 1.0 2.1 1.1 0.9 1.1 1.0	30
LT-3	thr(ser$_2$,asp,tyr,lys)	
	moles 1.0 1.9 1.1 0.5 1.0	55
LT-4	tyr(leu,asp,ser,arg)	
	moles 0.8 1.0 1.2 1.0 0.6	95
LT-5A	his(ser,glu,gly,thr,phe)	
	moles 0.5 1.0 1.0 0.9 1.0 1.2	30
LT-5B	arg	80
LT-PB	ala(glu$_2$,asp$_2$,phe,val,try,[b] leu,met,thr)	
	moles 1.2 2.1 1.9 1.1 1.0 1.0 1.1 0.6 0.9	~30

[a] The amino acids in all the peptides were analyzed as DNP-derivatives,[4] with the exception of LT–PB, which were determined by resin chromatography.[6] [b] Chemical analysis.[12]

As previously discussed, the rate of hydrolysis data indicate that two major splits occurred during the first few hours of incubation. At the end of 50 hr. digestion, three additional partial splits were observed. This complicated situation is not conducive to the clear analysis that is possible when bonds are completely cleaved. Nevertheless it is of interest to consider the isolated products in the light of the splits predictable from the rate study.

When the yield and composition of the fragments are considered, only one peptide from the 50 hr. digest, LT-4, was found to be similar to a 2.25 hr. digestion product. Presumably the other major fragments isolated from the 2.25 hr. incubate, ST-3 and ST-PA, must have been further degraded.

The sum of the amino acids in peptides LT-5A and LT-3 is identical to the amino acid composition of ST-3. Apparently the trypsin preparation split ST-3 in the following manner

his(ser,glu,gly,thr,phe,thr,ser,asp,tyr,ser,lys)

↓

his(ser,glu,gly,thr,phe) + thr(ser,asp,tyr,ser,lys)

This hydrolysis is quite different from that usually produced by trypsin, as no basic amino acid is involved.

The total amino acid content of fragments LT-1, LT-2 and LT-5A is the same as that of ST-PA. The release of arginine (LT-5B or ST-2) from the N-terminus of ST-PA may possibly be attributed to trypsin. However, the additional rupture of a bond in ST-PA to form LT-1 and LT-2 does not fit the accepted specificity, since no basic residues can be involved. The further splitting of ST-PA may be depicted as follows:

arg(ala,glu,asp,phe,val,glu,try,leu,met,asp,thr)

↓

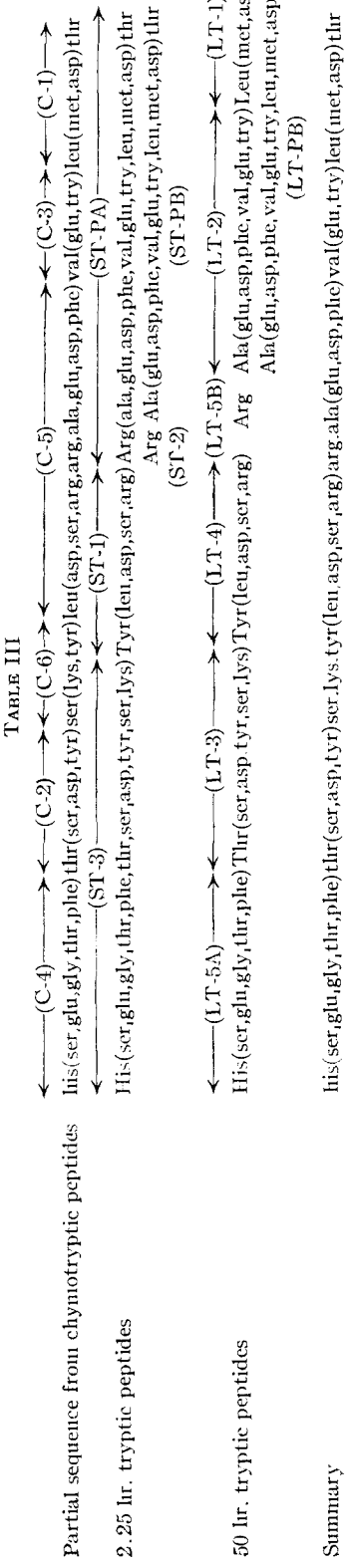
arg + ala(glu,asp,phe,val,glu,try) + leu(met,asp,thr)

Thus, a trypsin preparation has split two bonds in glucagon in addition to those predicted from the known specificity of trypsin. These observations are only incidental to the determination of the structure; nevertheless they are of considerable interest since nearly all other reports have confirmed the accepted specificity of trypsin. Two alternatives are possible; the activity is inherent in the trypsin, or it is caused by a proteolytic impurity such as chymotrypsin. The specificity of the hydrolysis is consistent with the latter interpretation; however, there is virtually no chymotrypsin in the trypsin preparation, only 2 of the known 5 chymotryptic splits were found, and the addition of β-phenyl propionate, a chymotrypsin inhibitor, to the incubate did not prevent the two splits in question.

Discussion

The information obtained from the tryptic digestion studies may be considered with respect to the structure of glucagon. Earlier work has shown that histidine and threonine are the N- and C-terminal residues of this chain, and that single residues of histidine, glycine, lysine, alanine, valine, tryptophan and methionine are present.

TABLE III



The unique amino acid composition of peptide ST-3 may be utilized to locate the position in the glucagon chain of two peptides from the chymotrypsin digestion, C-2 and C-6.

(ST-3)
his(ser,glu,gly,thr,phe,thr,ser,asp,tyr,ser,lys)
(C-4) (C-2) (C-6)
his(ser,glu,gly,thr,phe)thr(ser,asp,tyr)ser(lys,tyr)

All other evidence is consistent with this interpretation (*cf.* Table III).

The three major fragments of glucagon formed after 2.25 hr. of trypsin digestion, ST-3, ST-1 and ST-PA, may be readily placed in position within the glucagon molecule. Peptide ST-3 contains N-terminal histidine, and ST-PA contains all of the amino acids liberated by carboxypeptidase, including potential C-terminal threonine which is not found in ST-1. Hence, ST-3 and ST-PA must be the N- and C-terminal peptides, respectively. This leaves only a position between these fragments for ST-1, producing a sequence of (ST-3)(ST-1)(ST-PA). As previously discussed, these three peptides account for the entire amino acid composition of glucagon. Since ST-2 (arginine) and ST-PB were, in all likelihood, formed by the degradation of ST-PA, the sequence involving these minor products can only be written as (ST-3)(ST-1)(ST-2)(ST-PB), again accounting for the amino acid composition of glucagon.

Extending these considerations to the peptides isolated from the 50 hr. trypsin digest, it may be recalled that LT-5A and LT-3 were formed from ST-3, and LT-5B, LT-2 and LT-1 were formed from ST-PA. Peptide LT-4 is identical to ST-1, hence the sequence of peptides can only be written (LT-5A)(LT-3)(LT-4)(LT-5B)(LT-2)(LT-1). LT-PB, isolated in small yield and identical in composition to ST-PB from the 2.25 hr. digestion, can be located in a similar position.

The evidence for the sequence of the peptides within the glucagon chain is summarized in Table III.

Acknowledgment.—The authors gratefully acknowledge the paper chromatographic work of H. L. Bird and C. T. Pugh as well as the technical assistance of W. A. Tandy and R. G. Scheib.

INDIANAPOLIS 6, INDIANA

[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

The Amino Acid Sequence of Glucagon. IV. The Hydrolysis of Glucagon with Subtilisin

BY L. G. SINN, OTTO K. BEHRENS AND W. W. BROMER

RECEIVED NOVEMBER 19, 1956

Ten of the peptide bonds of glucagon were ruptured by the proteolytic attack of subtilisin. The resulting mixture of eleven degradation products was fractionated by Dowex 50 chromatography and countercurrent distribution. The arrangement of the peptide fragments in the glucagon chain is correlated with existing information.

Introduction

Previous reports[1,2] have described the hydrolytic breakdown products of glucagon obtained from the action of chymotrypsin and trypsin. Each set of peptides isolated from an enzymatic digest has an amino acid composition identical to that of glucagon. The peptides can be arranged logically in only one manner. Since some areas of unknown sequence remained, glucagon was split by use of a third proteolytic enzyme, subtilisin. The eleven peptides formed by this digestion are characterized in this report.

Experimental

Materials.—Twice-recrystallized glucagon, Lot 208-158B-292A, and crystalline subtilisin[3] (preparation F, containing 20% Na_2SO_4) were used for all experiments.

Hydrolysis of Glucagon by Subtilisin.—Glucagon (0.09%) was incubated with subtilisin (0.003%) at 37° in 0.05 *M* sodium acetate solution at *p*H 7.5 for 24 hr. During the first few hours of the digestion the *p*H drifted slowly to 7, and required occasional readjustment.

The rate and nature of the hydrolysis was studied by dinitrophenylating aliquots of the digest that were removed at various time intervals. The DNP-peptides were hydrolyzed, and the DNP-amino end groups of each new peptide were determined quantitatively.[4] In the preparative experiments the reaction mixture was immediately subjected to Dowex 50-X2 chromatography as described in a previous report.[1]

Results

Rate of Hydrolysis.—The rate of hydrolysis of glucagon by subtilisin is represented by the curves in Fig. 1. The amount of the N-terminal amino acid of each newly formed peptide is plotted *versus* time. The specificity of subtilisin has not been rigidly established[5]; however, it is certain that the enzyme ruptures a rather wide variety of peptide linkages, forming small peptide fragments. According to the data in Fig. 1 subtilisin almost completely split three bonds involving aspartic acid, two involving leucine, and one each involving glycine, valine and threonine. In addition, sizable amounts of DNP-arginine and di-DNP-lysine were detected, but were not determined quantitatively. Bonds involving these residues were ruptured to a significant extent. A logical interpretation of these data was that glucagon was split by subtilisin in 10 places, giving rise to 11 peptide fragments.

The splitting of most of the bonds was essentially complete within 5 to 6 hr.; however, some of the

(1) Reference to Paper II of this series, THIS JOURNAL, **79**, 2798 (1957).

(2) Reference to Paper III of this series, *ibid.*, **79**, 2801 (1957).

(3) We gratefully acknowledge receipt of subtilisin from Dr. M. Ottesen, Carlsberg Laboratories, Copenhagen, Denmark.

(4) Dinitrophenylations and quantitative analyses were performed according to the method of A. L. Levy, *Nature*, **174**, 126 (1954).

(5) H. Tuppy, *Monatsh.*, **84**, 996 (1953).