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).

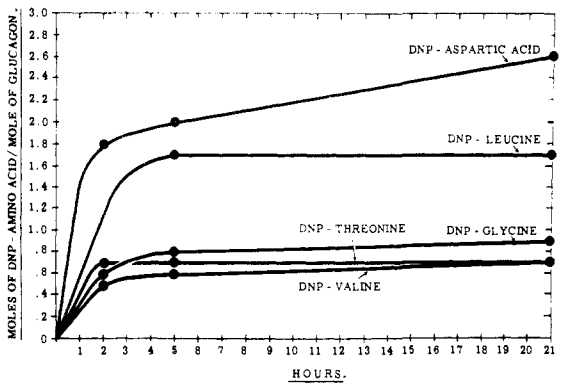

Fig. 1.—A study of the rate of the subtilisin hydrolysis of the peptide bonds of glucagon, as determined by the dinitrophenylation method. An 0.09% solution of glucagon was digested with subtilisin (0.003%) at *p*H 7.5 and 37°.

bonds required as long as 24 hr. for complete hydrolysis. Furthermore, since an uncertainty existed regarding the rate of splitting of arginine and lysine bonds, the incubation was continued for 24 hr. in the preparative experiments.

Separation and Analysis of the Peptides.—The Dowex 50-X2 effluent curve from the 24 hr. subtilisin digestion of 176.2 mg. (50.3 μmoles) of glucagon is presented in Fig. 2. The analysis and recovery of the peptide fragments is given in Table I.

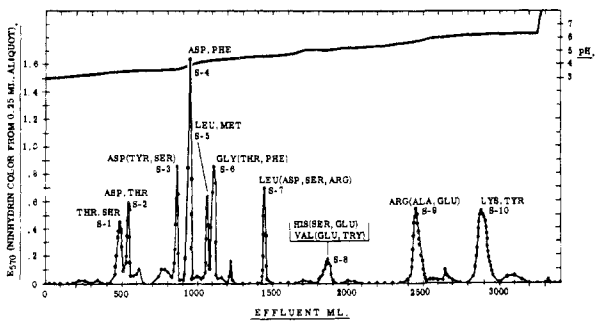

Fig. 2.—Separation of the degradation products resulting from the 24 hr. subtilisin digestion of 176.2 mg. of glucagon. Chromatography was performed on a 1.5 × 50 cm. column of Dowex 50-X2 resin. Five-ml. effluent fractions were collected. A 0.2 *N* NaOH solution was used to clean the resin column, producing the final sharp rise in *p*H of the effluent.

Peak no. 8 in the effluent curve in Fig. 2 was found to represent a mixture of two peptides. Upon application of the dinitrophenylation method both histidine and valine were identified as N-terminal residues. The DNP-derivatives of these peptides were separated by a 10 tube countercurrent distribution in 0.1 *N* HCl and ethyl acetate. The recovery of these two peptides was calculated after complete separation; the low yields were due, at least in part, to the increased number of manipulations. Both peptides, S-8A and S-8B, produced low color yields when reacting with ninhydrin, emphasizing the necessity for careful examination of all fractions which reacted with ninhydrin.

(6) J. R. Spies and D. C. Chambers, *Anal. Chem.*, **20**, 30 (1948).

TABLE I

PEPTIDES FROM THE SUBTILISIN DIGESTION OF GLUCAGON

Peptide no.	Peptide Compn. and anal.[a]	Yield, %	Peptide no.	Peptide Compn. and anal.[a]	Yield, %	Peptide no.	Peptide Compn. and anal.[a]	Yield, %
S-1	thr.ser	55	S-5	leu.met	60	S-8B	val(glu.try[b])	15
	moles 0.8 1.2			moles 1.1 0.9			moles 1.0 1.0 1.0	
S-2	asp.thr	80	S-6	gly(thr,phe)	85	S-9	arg(ala,glu)	60
	moles 1.1 1.0			moles 1.1 0.9 1.0			moles 1.2 0.9 1.0	
S-3	asp(tyr,ser)	50	S-7	leu(asp,ser,arg)	80	S-10	lys.tyr	75
	moles 1.0 1.0 1.1			moles 1.0 0.9 1.0 1.0			moles 1.0 1.0	
S-4	asp.phe	70	S-8A	his(ser,glu)	25			
	moles 1.1 1.0			moles 0.8 1.0 1.3				

[a] All peptides analyzed by paper chromatographic means. Results given are the averages of at least three analyses. [b] Chemical analysis.[6]

TABLE II

Proposed partial sequence	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
Peptides from subtilisin digest	S-8A S-6 S-1 S-3 S-10 S-7 S-9 S-4 S-8B S-5 S-2 His(ser,glu)Gly(thr,phe)Thr.ser Asp(tyr,ser)Lys.tyrLeu(asp,ser,arg)Arg(ala,glu)Asp.phe Val(glu,try)Leu.met Asp.thr
Summary	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

Paper chromatographic analyses of acid hydrolyzates of such fractions gave no evidence for peptide material other than the 11 peptides indicated in Table I.

With the methods used in this structure study, peptides might be overlooked that did not produce color with ninhydrin reagent. This possibility in itself is rather improbable. Furthermore, from the data on the rate of hydrolysis it has been possible to rather accurately predict the number of peptides formed in each incubation and the N-terminus of each peptide.

Discussion

Assuming that each peptide occurs only once in the chain, the distribution of the amino acids in the 11 peptides was identical to the amino acid composition of glucagon. Only peptide S-3, asp(tyr,leu), contained a measurable amount of impurity, having about 0.2 mole of threonine per mole of peptide. Dinitrophenylation and quantitative amino acid data, along with the behavior of the peptides on Dowex 50 columns and their generally good recovery, all pointed to the conclusion that these peptides were genuine fragments of glucagon arising from the hydrolytic action of subtilisin.

Integration of the 11 peptides from the subtilisin digestion into the glucagon chain is represented in Table II.

Acknowledgment.—The authors are indebted to H. L. Bird and C. T. Pugh for the paper chromatography, and to W. A. Tandy and R. G. Scheib for their technical assistance.

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[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

The Amino Acid Sequence of Glucagon. V. Location of Amide Groups, Acid Degradation Studies and Summary of Sequential Evidence

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

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Data is presented that elucidates the remaining unknown amino acid sequences in the glucagon chain, and that establishes the locations of the four amide groups. The following complete amino acid sequence of glucagon is proposed: 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

In preceding papers of this series[1-3] the degradation products of glucagon formed by the action of three different proteolytic enzymes have been described. These fragments of the glucagon chain can be arranged to fit together logically in only one manner; all of the data obtained are consistent with the proposed arrangement. However, the sequence of all the amino acids within the chain is not determined, and the location of the four amide groups can only be postulated from the behavior of the peptides on resin columns and from the carboxypeptidase data. This paper describes the elucidation of the unknown sequences, largely through partial acid degradation, and the final location of the amide linkages by means of chemical analysis.

Experimental

Partial Acid Degradation.—Peptides from enzymatic degradation of glucagon were dissolved in 11.7 *N* HCl at a concentration of about one μmole per ml., and the reaction mixture was incubated at 37° for 3 days. The excess acid was removed *in vacuo* over KOH pellets. The white residue was dissolved in water, and aliquots were subjected to paper chromatography in the solvent *n*-butanol:acetic acid:water, 4:1:1. The peptide degradation products were located by spraying an identification lane with ninhydrin reagent (*cf.* Fig. 1). Areas in the unreacted lanes corresponding to the ninhydrin-positive areas were excised, were eluted with 0.02 *N* HOAc, and were dinitrophenylated. The DNP-derivatives were hydrolyzed in acid, and the N-terminal residue and the amino acid composition were determined by paper chromatography.

Amide Analysis.—Two to 5 μmoles of a selected peptide was dissolved in dilute alkali at *p*H 8.5 to 9, and the solution was evaporated to dryness *in vacuo* over H_2SO_4. A solution of approximately 2 μmoles of peptide per ml. of 11.7 *N* HCl was prepared and was incubated at 37° for as long as 144 hr. At appropriate intervals, aliquots corresponding to 0.2 to 1 μmole of peptide were removed for ammonia analysis conducted either according to the method of Rees[4] or the procedure of Russell.[5] Each of the 7 peptides which were studied contained only one potential amide linkage.

In all cases a mixture of amino acids devoid of amide groups but otherwise simulating the peptide was treated similarly. Such control hydrolyses provided evidence for the amount of interfering substances or non-amide ammonia liberated.

Hydrolysis of Peptide C-3 with Carboxypeptidase.—A solution containing 0.02% peptide C-3, val(glu,try), and 0.024% carboxypeptidase was incubated under conditions identical to those employed in a previous report[6] enzyme: substrate mole ratio, about 1:65. Aliquots of the incubate were removed at various time intervals for dinitrophenylation and subsequent paper chromatography.[7]

Results

Partial Acid Degradation.—Three of the four remaining sequences were determined by characterizing the partial degradation products of the two enzymatically obtained peptides C-4, his(ser,glu)-

(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) Reference to Paper IV of this series, *ibid.*, **79**, 2805 (1957).

(4) M. W. Rees, *Biochem. J.*, **40**, 632 (1946).

(5) J. A. Russell, *J. Biol. Chem.*, **156**, 457 (1944).

(6) Reference to Paper I of this series, THIS JOURNAL, **79**, 2794 (1957).

(7) A. L. Levy, *Nature*, **174**, 126 (1954).