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.

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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 Received November 19, 1956

Data is presented that elucidates the remaining unknown amino acid sequences in the glucagon chain, and that establishes NH

the locations of the four amide groups. The following complete amino acid sequence of glucagon is proposed: His.ser.glu.-NH₂ NH₂ NH₂ NH₂

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 series¹⁻³ 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 μ mole 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

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

0.02 N HOAc, and were dinitrophenylated. The DNPderivatives were hydrolyzed in acid, and the N-terminal residue and the amino acid composition were determined by paper chromatography.

Anide Analysis.—Two to 5 μ moles of a selected peptide was dissolved in dilute alkali at ρ H 8.5 to 9, and the solution was evaporated to dryness in vacuo over H₂SO₄. A solution of approximately 2 μ moles of peptide per ml. of 11.7 N HCI was prepared and was incubated at 37° for as long as 144 hr. At appropriate intervals, aliquots corresponding to 0.2 to 1 μ mole of peptide were removed for ammonia analysis conducted either according to the method of Rees⁴ 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

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 report⁶ 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)-

(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).

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

⁽³⁾ Reference to Paper IV of this series, ibid., 79, 2805 (1957).



Fig. 1.—Photographs of the paper chromatographic separation of the acid hydrolysis products from peptides C-4, LT-3 and LT-4. The chromatograms were developed with *n*-butanol:acetic acid:water (4:1:1), and were treated with nin-hydrin reagent: A, hydrolysis products of peptide C-4, His(ser,glu,gly,thr,phe); B, hydrolysis products of peptide LT-3, Thr(ser,asp,tyr,ser,lys); C, hydrolysis products of peptide LT-4, Tyr(leu,asp,ser,arg).

gly(thr,phe), and LT-4, tyr.leu(asp,ser,arg). In addition, peptide LT-3, thr.ser.asp.tyr,ser.lys, was similarly degraded. The quantitative amino acid composition of these peptides had been determined in the earlier described work.^{1,2} Since acid degradation is not considered to promote rearrangements, such data elucidated the unknown sequences and also provided additional evidence for certain sequences which were proposed largely on the basis of data obtained from enzyme degradation.

Presented in Fig. 1 are photographs of the paper chromatographic separation of the acid hydrolysis products of the above three peptides.

The chromatograms were treated with ninhydrin reagent. In cases where overlapping of the spots occurred, the chromatograms were carefully excised to exclude the overlapping area. The analysis and recovery of the degradation products are presented in Table I. The figures on amino acid analysis are based on single or duplicate paper chromatographic analyses. Precise analytical data were not required to ascertain the amino acid composition of the degradation products since the parent peptides had been carefully analyzed and only one, LT-3, contained more than one residue of a given amino acid.

Small quantities of impurities were present in some of the hydrolytic products; this observation had been expected because of the non-specific degradative approach. Estimation of the purity of the fragments was based on end group analysis using the dinitrophenylation method. In the majority of cases at least 0.4 mole of a given amino acid residue was recovered per mole of starting peptide.

This recovery was considered acceptable since large losses had been expected due to the lengthy manipulation consisting of partial acid hydrolysis,

paper chromatogra	aphy,	elutio	on f	from	the	paper	, di-
nitrophenylation,	com	olete	aci	d h	ydro	lysis	and

	TABLE I	
PAPER	Chromatographic Analysis of the Acid Degrada	-
	TION PRODUCTS	

No. of parent peptide	Compn. and analysis of acid degrad. products	R_{f}	Esti- mated purity %	Major impurity
C-4	his	0.04	90	his(ser,glu)?
C-4	ser(glu,gly) moles 1.2 1.3 0.6	.12	80	gly
C-4	glu.gly moles 1.2 0.8	.20	100	
C-4	thr.phe moles 1.1 0.9	.60	100	
LT-3	ser.ly s moles 1.0 1.0	.05	80	lys
LT-3	ser.asp moles 1.1 0.9	.14	80	asp
LT-3	thr	.21	100	
LT-3	ser(asp,tyr) qual.	.37	65	asp(tyr.?)
LT-3	asp.tyr moles 1.2 0.8	. 50	100	
LT-4	ser.arg moles 0.9 1.1	.07	90	arg
LT-4	asp.ser qual.	.15	100	5 ×5
LT-4	tyr	.41	100	8. ANN
LT-4	leu.asp moles 0.8 1.2	.60	100	3 X.9
LT-4	tyr(leu,asp) moles 1.0 0.6 1.4	.72	100	
LT-4	tyr.leu moles 1.0 1.0	.86	100	

paper chromatographic analysis of both the free and the DNP-amino acids. The degradation of the parent peptides into relatively few fragments may be attributed at least in part to the high content of serine and threonine in the peptides. Bonds involving the amino groups of the hydroxy amino acids are known⁸ to be particularly labile in concentrated acids at rather low temperatures.

Sequence of Peptide C-3.—Paper chromatography⁷ of the dinitrophenylated aliquots from the carboxypeptidase time study of peptide C-3 revealed the presence of two major DNP-derivatives in approximately equal amount. DNPtryptophan was recognized readily; however, the second derivative did not correspond to any known DNP-amino acid. Quantitative analysis⁹ demonstrated the complete absence of tryptophan in the unknown DNP-derivative. After hydrolysis, followed by paper chromatographic separation, the unknown spot was identified as DNP-val.glu. The course of the hydrolysis is represented by the curves in Fig. 2, with the DNP-peptide calculated as if it



Fig. 2.—The rate of hydrolysis of peptide C-3, val(glu, try), by carboxypeptidase as determined by the dinitro-phenylation method.

were DNP-valine. While the quantitative aspects of this experiment leave something to be desired, there can be little doubt that the sequence in peptide C-3 is val.glu.try. Both products of hydrolysis point to this conclusion; furthermore, this conclusion is in agreement with the usual specificity of chymotrypsin.

Location of the Amide Linkages.—The curves presented in Fig. 3 are representative of the results obtained from the amide analysis of peptides C-3, C-4, S-2, S-3, S-4, S-7 and S-9. In some cases the zero-time values for ammonia were abnormally high despite efforts to remove extraneous ammonia by concentration to dryness *in vacuo* from a weakly basic solution. Because of this difficulty it is possible that the quantitative recovery of the ammo-

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



Fig. 3.—Amide analysis of peptides S-2 and S-3: A, ammonia nitrogen liberated from $1.2 - \mu M$ aliquots of peptide S-2, and from equal quantities of a mixture of aspartic acid and threenine. Extrapolation to zero time provides a value of approximately one μM of NH₃ formed per μM of peptide S-2. B, Ammonia nitrogen liberated from 0.2 μM -aliquots of peptide S-3. Less than 0.1 μM of NH₃ was formed per μM of peptide S-3.

nia was masked by the initial high value, particularly in the cases of peptides C-4 and S-9

Despite this factor, the gradual release of ammonia during the digestion clearly showed which peptides contained the amide linkages. The quantitative data are presented in Table II. Of the 7

TABLE II

AMIDE ANALYSIS OF PEPTIDES FROM GLUCAGON DEGRADA-

	11014	
Peptide no.	Peptide compn.	Moles of amide pe mole of peptide
	NH_2	
C-3	Val(glu,try) NH ₂	1.1
C-4	His(ser glu gly thr phe)	0.6
0-1	NH ₂	0.0
S-2	Asp.thr	. 9
S-3	Asp(tyr,ser)	.03
S-4	Asp,phe	. 03
S-7	Leu(asp,ser,arg)	.0
	NH₂	
S-9	Arg(ala,glu)	.5

peptides tested, only C-3, C-4, S-9 and S-2 yielded significant quantities of ammonia when incubated with strong acid. This is in agreement with the number of amide linkages found by chemical analysis of glucagon, and with the behavior of the peptides on resin columns. The data presented earlier on the release of amino acids from glucagon by the action of carboxypeptidase are also consistent with the conclusions that have been presented. Three amide-containing residues were liberated.

⁽⁸⁾ F. Sanger, Advances in Protein Chem., 7, 1 (1952).

Proposed partial acquares Ander groups Ander groups Acid acgroups Acid A			TABLE	111				
Amide groups and set of the set as point of the set aset of the set as point of the set as point of the se	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						
Amide groups A mide groups A m	Curson, peptidase	NH_2			NH2	NH2	NH2	
peptides ser(glu.gly) ser(ap.tyr) tyrileu ser(ap.glu.gly) ser asp thr.phe ser,ap ap.ser ser.arg NHa NHa NHa NHa Summary Hisser.glu.gly.thr.phe.thr.ser.asp.tyr.ser.lys.tyr.leu.asp.ser arg.ala.glu.asp.phe.val.glu.try.leu.met.asp.thr TABLE IV SUMMARY OF EVIDENCE LEADING TO THE AMINO ACID SEQUENCE OF GLUCAGON Peptide no. ⁴ Degradation products ST.3 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 Alis(ser.glu.gly.thr.phe,thr.ser.asp.tyr.ser.lys.tyr.leu.asp.ser.arg.tr Stanson acids liberated by Anino acids liberated by NHa NHA Anino acids liberated by NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA S.2 ST.3 C.2 Stanson acids liberated by NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA NHA NH	Amide groups Acid degradation of certain	His(ser,glu,gly,thr,phe) His tl	asp(tyr,ser) 1r	leu(asp,ser,arg)a tvr.	arg.(ala,glu)asp.	phe val (glu,try	r) asp.thr	
ser.lys ser.arg ser.arg NH1 NH2 NH3 NH4 ST-3 His(ser glu.gly, thr.phe, thr.ser.asp, tyr.ser.lys SA His (ser glu.gly, thr.phe SA SA SA His (ser glu.gly, thr.phe SA SA SA SA SA S	peptides	ser(glu.gly) glu.gly thr.phe	ser(asp,tyr) ser.asp asp,tyr	tyr.leu tyr(leu,asp) leu.asp				
NHr NHr NHr NHr NHr Summary Hisser 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 TABLE IV SUMMARY OF EVIDENCE LEADING TO THE AMINO ACID SEQUENCE OF GLUCAGON Peptide no.* Degradation products ST-3 Mis(ser glu.gly.thr.phe.thr.ser.asp.tyr.ser.lys) S4 NH LT-5A His(ser glu.gly.thr.phe S6 S6 S1 S3 S4 Thr.ser S6 S1 S7 S1 S7 S6 S1 S7 S1 S1 S1 S2 S1 S2 S2 <td></td> <td></td> <td>ser.l</td> <td>ys asp.ser ser.arg</td> <td></td> <td></td> <td></td>			ser.l	ys asp.ser ser.arg				
Summary Hisser, glugy, thr. phe. thr. ser. asp. tyr. ser. Jys. tyr. leu. asp. ser. arg. arg. ala. glu. asp. phe. val. glu. try. leu. met. asp. thr TABLE IV CMMMARY OF EVIDENCE IE LADINO ACID SEQUENCE OF GLUCAGON Peptide no.* Degradation products ST-3 Hisser glugy, thr. phe. thr. ser. asp. tyr. ser. Jys SA Hisser glugy, thr. phe. thr. ser. asp. tyr. ser. Jys S-3 C-4 IIII IIIIIIIIIIIIIIIIIIIIIIIIIIIIIII		NH2		0	NH₂	NH2	NH2	
TABLE IV Depride no.* Depride no.* Depride no.* ST-3 Hister glu.gly.thr.phe,thr.ser.asp.tyr.ser.lys Set Depride no.* Set S-8.4 Hister.glu Set <	Summary	His.ser.glu.gly.thr.phe.th	.ser.asp.tyr.ser.ly	s.tyr.leu.asp.ser.arg.a	rg.ala.glu.asp.pl	1e.val.glu.try.le	u.met.asp.thr	
SUMMARY OF EVIDENCE LEADING TO THE AMINO ACID SEQUENCE OF GLUCAGON Peptide no. ^a Degradation products Peptide no. ^a Degradation products S43 His(ser glu.gly, thr.phe, thr.ser, asp.,tyr.ser, lys) S44 NH, C4 NH, L7-5A His(ser.glu.gly, thr.phe S6 gly(thr.phe) L7-5A His.ser.glu.gly, thr.phe S6 gly(thr.phe) S1 thr.ser.asp.,tyr.ser.lys S2 thr.ser.asp.,tyr.ser.lys S2 thr.ser.asp.,tyr.ser.lys S2 thr.ser.asp.,tyr.ser.lys S2 thr.ser.asp.,tyr.ser.lys <td></td> <td></td> <td>TABLE</td> <td>IV</td> <td></td> <td></td> <td></td>			TABLE	IV				
Peptide no. ^a Degradation products ST.3 His(ser_glu) St.4 His(ser_glu) C-4 NH, LT-5A His(ser_glu,gly,thr.phe) S-6 gly(thr.phe) S-7 gly(thr.phe) S-1 thr.ser.asp.tyr.ser.lys C-2 thr.ser.asp.tyr.ser.lys S-3 thr.ser.asp.tyr.ser.lys S-4 thr.ser.asp.tyr.ser.lys S-5 saft(tr,ser) S-6 saft(tr,ser) C-2 thr.ser.asp.tyr.ser.lys S-1 thr.ser.asp.tyr.ser.lys S-6 saft(tr,ser) S-7 leu(asp.ser.arg) S-7 leu(asp.ser.arg, and, aglu, asp.phe) S-7 leu(asp.ser.arg, arg, ala, glu, asp.pheval.glu, try.leu,met, asp)thr S-7 leu(asp.ser.arg, arg, ala, glu, asp.pheval.glu, try.leu,met, asp)thr S-7 leu(asp.ser.arg, arg, ala, glu, asp.pheval.glu, try.leu,met, asp)thr S-7 aarg(ala, glu, asp.pheval.glu, try.leu,met, asp)thr S-7 ala(glu, asp.pheval.glu, try.leu,met, asp)thr S-7 saft	Si	UMMARY OF EVIDENCE LE	ADING TO THE A	Amino Acid Sequi	ence of Gluc	AGON		
ST-3 His(ser glu,gly,thr,phe,thr,ser,asp,tyr,ser,lys) S-8A His(ser,glu) S-8A His(ser,glu) S-8A His(ser,glu,gly,thr,phe) S-6 gly(thr,phe) S-6 gly(thr,phe) S-7 thr ser (sp, styr) S-1 thr(ser,asp,tyr) S-3 asp(try,ser) C-6 ser(1ys,tyr) S-10 tyr,teu.asp.ser.arg S-7 leu(asp,ser,arg) C-5 leu(asp,ser,arg) C-5 leu(asp,ser,arg) S-7 ser(3a,slu,asp,phe,val,glu,try,leu,met,asp)thr <td< td=""><td>Peptide no.ª</td><td></td><td>1</td><td>Degradation products</td><td>ł</td><td></td><td></td></td<>	Peptide no.ª		1	Degradation products	ł			
S-8A His(ser.glu) C-4 NH LT-5A His.ser.glu.gly,thr.phe S-6 gly(thr.phe) LT-3 thr.ser C-2 thr.ser C-2 thr.ser C-2 thr.ser C-3 asp(tyr,ser) S-6 ser(1ys,tyr) S-10 lys,tyr S-7 leu(asp.ser.arg) C-5 leu(asp.ser.arg,arg,ala,glu,asp.phe) ST-2 arg(ala,glu) ST-2 arg(ala,glu)	ST-3	His(ser glu gly throhe thro	er osp tvr ser lvs)					
C-4 NH3 LT-5A Hisser, glu, gly, thr.phe S-6 gly(thr,phe) LT-3 thr.ser, glu, gly, thr.phe S-6 gly(thr,phe) S-1 thr.ser, asp.tyr) S-3 thr(ser, asp.tyr) S-3 sap(tyr, ser) S-6 sclosed by styr S-7 leu(asp.ser, arg) C-5 leu(asp.ser, arg) C-5 leu(asp.ser, arg, arg, ala, glu, asp, phe) ST-2 arg (ala, glu, asp, phe, val, glu, try, leu, met, asp) thr ST-2 ala (glu, asp, phe, val, glu, try, leu, met, asp) thr ST-PA arg (ala, glu, asp, phe, val, glu, try, leu, met, asp) thr ST-PA ala (glu, asp, phe, val, glu, try, leu, met, asp) thr ST-24 ala (glu, asp, phe, val, glu, try, leu, met, asp) thr ST-PA ala (glu, asp, phe, val, glu, try, leu, met, asp) thr S-5 closed val, glu, try, leu, met, asp) thr S-6 closed val, glu, try, leu, met, asp) thr S-7 closed val, glu, try, leu, met, asp) thr S-8 closed val, glu, try, leu, met, asp) thr S-2 closed val, glu, try, leu, met, asp) thr NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4	S-8A	His(ser.glu)	er,asp,cy1,ser,rys,					
LT-5A His.ser_glu.gly.thr.phe S6 gly(thr.phe) LT-3 thr.ser.asp.tyr.ser.lys S-1 thr.ser.asp.tyr) S3 thr.ser.asp.tyr) S3 thr.ser.asp.tyr) S3 thr.ser.asp.tyr) S3 thr.ser.asp.tyr) S10 lys.tyr S10 lys.tyr S10 lys.tyr S7-1 tyr.leu.asp.ser.arg S7 leu(asp.ser.arg,arg,ala,glu,asp,phe) S7 leu(asp.ser.arg,arg,ala,glu,asp,phe) S7 leu(asp.ser.arg,arg,ala,glu,asp,phe) S7 leu(asp.ser.arg,arg,ala,glu,asp,phe,val,glu,try,leu,met,asp)thr S7 leu(asp.phe,val,glu,try,leu,met,asp)thr S7-PA arg(ala,glu,asp,phe,val,glu,try) S4 NH4 leu(met,asp)thr S4 leu(met S-2 leu(met,asp)thr S-2 leu(met S-2 leu(met S-3 leu(met S-3 laglu,asp,phe,val,glu,try) S4 asp.thr NH4 NH4 NH4 NH4 NH4 Amino acids liberated by liter NH4 NH4 NH4 NH4 NH4 NH4	C-4	NH,						
S6 . gly(thr,phe) LT-3 thr.ser.asp.tyr.ser.lys S1 thr.ser C-2 thr(ser,asp,tyr) S3 ser(lys,tyr) S10 lys,tyr S10 lys,tyr S10 lys,tyr S11 thr(ser,asp,tyr) S10 lys,tyr S11 thr(ser,asp,tyr) S10 lys,tyr S11 thr(ser,asp,tyr) S10 lys,tyr S11 thr(ser,asp,tyr) S12 thr(ser,asp,tyr) S13 thr(ser,asp,tyr) S14 server S14 server S15 server S15 server S17 server S19 server S20 server S19 server S20 server S19 server S20 server S19 server S20 server S19 server S20 serv	LT-5A	His.ser.glu.glv.thr.phe						
1.T-3 thr.ser.asp.tyr.ser.lys S.1 thr.ser C-2 thr(ser,asp.tyr) S.3 asp(tyr,ser) S-6 ser(lys,tyr) S.10 lys,tyr ST-1 lys,tyr S.7 leu(asp.ser.arg C-5 leu(asp.ser.arg,arg,ala,glu,asp,phe) ST-2 arg S.7 leu(asp.ser.arg,arg,ala,glu,asp,phe) ST-2 arg S.9 arg(ala,glu) ST-PB arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr S.4 ala(glu,asp,phe,val,glu,try) S.4 sep,phe C-3 val.glu.try S.8B NH4 S-5 leu(met,asp)thr C-1 leu(met,asp)thr LT-1 asp.thr S.2 NH4 Amino acids liberated by NH4 carboxypeptidase ala(glu,asp,phe,val,glu,try,leu,met,asp)thr NH4 NH4 NH4 NH4	S-6	glv(thr.nhe)						
S-1 three th	LT-3	8-5 (,p=c) thr.s	er asn fvr ser lvs					
C-2 three rap. tyr) S-3 asp(tyr, ser) S-6 ser(1ys, tyr) S-10 lys, tyr S-11 tyr.leu.asp.ser.arg LT-4 S-7 leu(asp, ser, arg) C-5 leu(asp, ser, arg) C-5 leu(asp, ser, arg, arg, ala, glu, asp, phe) ST-2 arg (ala, glu, asp, phe, val, glu, try, leu, met, asp) thr S-9 arg (ala, glu, asp, phe, val, glu, try, leu, met, asp) thr ST-PA arg (ala, glu, asp, phe, val, glu, try, leu, met, asp) thr LT-PB LT-2 ala (glu, asp, phe, val, glu, try, leu, met, asp) thr LT-2 ala (glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-4 break arg (ala, glu, asp, phe, val, glu, try) S-5 creak asp, phe S-2 creak asp, phe (alg, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try, leu, met, asp) thr NH4 break ala (glu, asp, phe, val, glu, try,	S-1	thr.s	er					
S-3 asp(tyr, set) C-6 ser(1ys, tyr) S-10 lys, tyr ST-1 tyr.leu.asp.ser.arg LT-4 tyr.leu.asp.ser.arg S-7 leu(asp, ser, arg, arg, ala, glu, asp, phe) ST-2 arg LT-5B N S-9 ST-PA arg(ala, glu, asp, phe, val, glu, try, leu, met, asp) thr ST-PA ala(glu, asp, phe, val, glu, try, leu, met, asp) thr ST-PB ala(glu, asp, phe, val, glu, try, leu, met, asp) thr LT-2 ala(glu, asp, phe, val, glu, try) S-4 NH ₄ leu.met C-3 val, glu, try S-8B S S-5 leu(met, asp) thr S-2 Aspect of the set of	C-2	three	er aso tvr)					
C-6 ser(1ys,tyr) S-10 lys,tyr S-11 tyr,leu,asp.ser,arg LT-4 tyr,leu,asp.ser,arg) C-5 leu(asp,ser,arg) ST-2 arg LT-5B N S-9 arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr S-9 arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr LT-PB ala(glu,asp,phe,val,glu,try) S-4 ala(glu,asp,phe,val,glu,try) S-5 leu(met C-3 ser(1-1) leu(met S-5 leu(met C-1 leu(met C-1 leu(met S-2 asp.thr Amino acids liberated by carboxypeptidase ala(glu,asp,phe,val,glu,try,leu,met,asp)thr NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 S-1 leu(met,asp)thr NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4	S-3		asp(tyr ser)					
S-10 [rs,tyr ST-1 tyr.leu.asp.ser.arg LT-4 (rs,tyr) S-7 leu(asp,ser,arg) C-5 leu(asp,ser,arg,arg,ala,glu,asp,phe) ST-2 arg S-5 leu(asp,ser,arg,arg,ala,glu,asp,phe) ST-2 arg S-9 (rs,targ,ala,glu) ST-PA (rs,targ,ala,glu) ST-PA (rs,targ,ala,glu) ST-PA (rs,targ,ala,glu) ST-PA (rs,targ,ala,glu) ST-PA (rs,targ,ala,glu) ST-PA (rs,targ,ala,glu) ST-PA (rs,targ,ala,glu) ST-PA (rs,targ,ala,glu) ST-PA (rs,targ,ala,glu,asp,phe,val,glu,try,leu,met,asp)thr ala(glu,asp,phe,val,glu,try) S-4 (rs,targ,ala,glu) S-4 (rs,targ,ala,glu) S-4 (rs,targ,ala,glu) S-4 (rs,targ,ala,glu,asp,phe,val,glu,try) S-4 (rs,targ,ala,glu) S-2 (rs,targ,ala,glu) S-2 (rs,targ,ala,glu,asp,phe,val,glu,try) S-2 (rs,targ,ala,glu) S-2 (rs,targ,ala,glu,asp,phe,val,glu,try) S-2 (rs,targ,ala,glu) S-2 (rs,targ,ala,glu) S-2 (rs,targ,ala,glu,asp,phe,val,glu,try) S-4 (rs,targ,ala,glu) S-2 (rs,targ,ala,glu) S-2 (rs,targ,ala,glu) S-2 (rs,targ,ala,glu,asp,phe,val,glu,try,leu,met,asp)thr NH ₄ (rs,targ,ala,glu,try,leu,met,asp)thr NH ₅ (rs,tar	C-6		ser(lve	tyr)				
ST-1 tyr.leu.asp.ser.arg LT-4 tyr.leu.asp.ser.arg S.7 leu(asp.ser.arg, arg, ala, glu, asp, phe) ST-2 arg LT-3B NH4 S.9 arg(ala, glu) ST-PA arg(ala, glu, asp, phe, val, glu, try, leu, met, asp) thr ST-PB ala(glu, asp, phe, val, glu, try, leu, met, asp) thr LT-2 ala(glu, asp, phe, val, glu, try, leu, met, asp) thr ST-PB ala(glu, asp, phe, val, glu, try) S.4 asp.phe C-3 val.glu, try S-5 leu(met C-1 leu(met LT-1 NH4 S-2 asp.thr Amino acids liberated by NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4	S-10		ser (rys,	tvr				
LT-4 ()) (Luap) Seriarg S.7 leu(asp,ser,arg) C.5 leu(asp,ser,arg) S.7 leu(asp,ser,arg, arg,ala,glu,asp,phe) ST-2 arg LT-5B "NH1" S.9 arg(ala,glu) ST-PA arg(ala,glu) ST-PB ala(glu,asp,phe,val,glu,try,leu,met,asp)thr LT-2 ala(glu,asp,phe,val,glu,try) S.4 NH1 C-3 val.glu.try S-5 leu.met C-1 leu(met,asp)thr LT-1 NH1 S-2 asp.thr Amino acids liberated by NH1 carboxypeptidase ala(glu,asp,phe,val,glu,try,leu,met,asp)thr	ST-1		195,	tyrleu opp oer org				
S-7 leu(asp,ser,arg) C-5 leu(asp,ser,arg,arg,ala,glu,asp,phe) ST-2 arg LT-5B NH1 S-9 arg(ala,glu) ST-PA arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr ST-PB ala(glu,asp,phe,val,glu,try,leu,met,asp)thr LT-7B ala(glu,asp,phe,val,glu,try) S-4 asp.phe C-3 val.glu,try S-8B leu(met,asp)thr S-5 leu(met,asp)thr C-1 NH1 LT-1 NH2 S-2 asp.thr Amino acids liberated by NH1 NH1 carboxypeptidase ala(glu,asp,phe,val,glu,try,leu,met,asp)thr NH4 NH4 NH4	LT-4			cyr.ieu.asp.ser.arg				
C-3 Ieu(asp,set,atg) ST-2 Ieu(asp,set,atg) LT-5B Ieu(asp,set,atg) S-9 arg ST-PA arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr ST-PB arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr LT-7B ala(glu,asp,phe,val,glu,try,leu,met,asp)thr LT-2 ala(glu,asp,phe,val,glu,try) S-4 asp,phe C-3 val.glu,try S-5 leu(met,asp)thr LT-1 NH1 S-2 asp,thr Amino acids liberated by NH2 NH1 NH3 NH1 NH3 NH1 NH3 NH1 NH3	S-7			leu (osp ser arg)				
ST-2 arg LT-5B NH1 S-9 arg(ala,glu,asp,ple,val,glu,try,leu,met,asp)thr ST-PA arg(ala,glu,asp,ple,val,glu,try,leu,met,asp)thr ST-PB ala(glu,asp,ple,val,glu,try,leu,met,asp)thr LT-2 ala(glu,asp,ple,val,glu,try) S-4 asp,ple C-3 val.glu.try S-5 leu.met C-1 leu.met LT-1 NH4 S-2 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4	C-5			leu(asp,ser,arg)	g ala glu asp ph	•		
LT-5 NHa S-9 arg(ala,glu) ST-PA arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr ST-PB ala(glu,asp,phe,val,glu,try,leu,met,asp)thr LT-2 ala(glu,asp,phe,val,glu,try) S-4 asp.phe C-3 val.glu.try S-5 leu.met C-1 leu(met,asp)thr LT-1 NHa S-2 asp.thr Amino acids liberated by NHa carboxypeptidase NHa NHa NHa	ST-2			reu(asp,ser,arg,ar,	a S'ara'Str'ssh'hue	3)		
S-9 arg(ala,glu) ST-PA arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr ST-PB arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr LT-2 ala(glu,asp,phe,val,glu,try) S-4 asp,phe C-3 sele selection of the selectio	LT-5B			al	S NH.			
S-9 arg(ala,glu) ST-PA arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr ST-PB ala(glu,asp,phe,val,glu,try,leu,met,asp)thr LT-PB ala(glu,asp,phe,val,glu,try) S-4 asp,phe C-3 val.glu.try S-5 val.glu.try C-1 leu(met,asp)thr LT-1 NH4 S-2 Amino acids liberated by carboxypeptidase NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4								
ST-PA arg(ala,glu,asp,phe,val,glu,try,leu,met,asp)thr ST-PB ala(glu,asp,phe,val,glu,try,leu,met,asp)thr LT-PB ala(glu,asp,phe,val,glu,try) S-4 asp,phe C-3 val.glu.try S-5 leu.met C-1 leu(met,asp)thr LT-1 NH1 S-2 asp,phe Amino acids liberated by NH1 NH2 carboxypeptidase NH2 i NH1 NH2 i NH2 i i S-2 NH2 i Amino acids liberated by NH2 i Carboxypeptidase NH2 i NH3 NH3 NH3 NH4 NH4 NH4	S-9			ar	g(ala,glu)			
ST-PB ala(glu,asp,phe,val,glu,try,leu,met,asp)thr LT-PB ala(glu,asp,phe,val,glu,try) S-4 asp.phe C-3 NH1 S-8B it S-5 leu.met C-1 leu(met,asp)thr LT-1 NH1 S-2 leu(met,asp)thr Amino acids liberated by NH1 it carboxypeptidase NH2 it NH2 it it NH2 it it NH2 it it S-2 NH2 it Amino acids liberated by NH2 it NH2 NH2 it NH2 NH2 it NH3 it it	ST-PA			ar	g(ala,glu,asp,ph	e,val,glu,try,l e u	,met,asp)thr	
LT-PB LT-2 ala(glu,asp,phe,val,glu,try) S-4 asp,phe C-3 val.glu.try S-8B S-5 C-1 leu.met C-1 LT-1 leu(met,asp)thr LT-1 NH ₁ NH ₁ NH ₁ S-2 Amino acids liberated by carboxypeptidase ala(glu,asp,phe,val,glu,try,leu,met,asp)thr NH ₁ NH ₁	ST-PB				ala(glu,asp,ph	e,val,glu, tr y,leu	,met,asp)thr	
LT-2 ala(glu,asp,phe,val,glu,try) S-4 asp.phe NH ₃ C-3 val.glu.try S-8B S-5 C-1 LT-1 S-2 Amino acids liberated by carboxypeptidase NH ₃ NH ₃ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH ₄ NH	LT-PB							
S-4 asp.phe NH1 C-3 val.glu.try S-8B leu.met S-5 leu(met,asp)thr C-1 NH1 LT-1 NH1 S-2 NH1 Amino acids liberated by NH1 carboxypeptidase NH2 NH3 NH3 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4 NH4	LT-2				ala(glu,asp,ph	e,val,glu,try)		
C-3 val.glu.try S-8B S-5 leu.met C-1 leu(met,asp)thr LT-1 NH ₃ S-2 Amino acids liberated by carboxypeptidase Amino acids liberated by liberated	S-4				asp.ph	e NH:		
C-3 val.glu.try S-8B S-5 leu.met C-1 leu(met,asp)thr LT-1 NHs S-2 Amino acids liberated by carboxypeptidase MHs NHs NHs NHs NHs ala(glu,asp,phe,val.glu,try,leu,met,asp)thr NHs NHs NHs NHs NHs NHs NHs NHs NHs NHs NHs NHs NHs NHs NHs						1		
S-8B leu.met S-5 leu(met,asp)thr LT-1 NH1 S-2 asp.thr Amino acids liberated by carboxypeptidase NH2 NH3 NH3 NH4 I NH4 I Image: S-2 Image: S-2 Amino acids liberated by carboxypeptidase NH3 Image: S-2 Image: S-2 Image: S-2 Image: S-2 <td>C-3</td> <td></td> <td></td> <td></td> <td></td> <td>val.glu.try</td> <td></td>	C-3					val.glu.try		
S-5 leu.met C-1 leu(met,asp)thr LT-1 NH1 S-2 asp.thr Amino acids liberated by NH1 NH2 carboxypeptidase NH2 NH2 NH2 Amino acids liberated by NH2 NH2 Amino acids liberated by Amino acids liberated by NH2 NH2 Image: NH2 NH2 NH2 NH2 NH2 NH3 Image: NH3 Image: NH3 Image: NH3 Image: NH3 Image: NH3 Image: NH3	S-8B							
C-1 LT-1 S-2 Amino acids liberated by carboxypeptidase NH2 NH2 NH2 NH2 NH2 NH2 NH2 NH2 NH2 NH2	S-5					1 e 1	1.met	
LT-1 S-2 Amino acids liberated by carboxypeptidase NH2 NH2 NH2 NH2 NH2 NH2 NH2 NH2 NH2 NH2	C-1					lei	(met,asp)thr	
S-2 Amino acids liberated by carboxypeptidase NH2 NH2 NH2 NH2 NH2 NH2 NH2 NH2	LT-1						NH:	
Amino acids liberated by NH1 NH2 NH2 carboxypeptidase NH2 NH2 NH2 NH2 NH2 NH3 I I I I	S-2						asp.thr	
NH1 I I NH2 NH2 NH3	Amino acids liberated by				NH3	NH3	NH3	
NH ₁ NH ₂ NH ₃ H	carboxypeptidase				j ala(glu asp. ph	i e val.glu.trvier	l met.asp)tbr	
		NH:			NH:	NH:	NH:	

Proposed sequenceHis.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.thra Prefix of the peptide number indicates the hydrolytic agent:ST = trypsin (2.25 hr.), S = subtilisin, C = chymotrypsin,LT = trypsin (50 hr.).

Two, as glutamine, occur in peptides C-3 and S-9, One, as asparagine, has been found in the terminal peptide, S-2. Finally, a residue of aspartic acid was released slowly by carboxypeptidase; the identification of this residue with the aspartic acid occurring in peptide S-4 is a logical interpretation.

Discussion

The results obtained by degradation of certain peptides with acid and with carboxypeptidase have been integrated into existing knowledge of the glucagon structure as shown in Table III. At least one line of evidence is available for each sequence in the chain, and in many cases more than one proof has been presented. In no case was it necessary to rely on the specificity of a proteolytic enzyme for a structure proof; however, with the exception of 2 or 3 minor splits caused by trypsin, the accepted specificity patterns of trypsin and chymotrypsin were confirmed. Of critical importance is the fact that all data, with no exceptions, are consistent with the final structure as proposed in Table III. A summary of all the evidence described in this series of reports is presented in Table IV.

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