[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

The Amino Acid Sequence of Glucagon. I. Amino Acid Composition and Terminal Amino Acid Analyses

BY W. W. BROMER, A. STAUB, E. R. DILLER, H. L. BIRD, L. G. SINN AND OTTO K. BEHRENS

RECEIVED NOVEMBER 19, 1956

Evidence is presented that glucagon is a small protein, consisting of a single chain of 29 amino acid residues. The N-terminal amino acid is histidine as determined by the dinitrophenylation method; the C-terminal residue is threonine on the basis of evidence obtained from hydrazinolysis and carboxypeptidase treatment. Glucagon contains single residues of 7 amino acids; among them, methionine, tryptophan, valine and alanine are liberated from the C-terminus of the molecule by carboxypeptidase.

Introduction

The preparation[1] in this Laboratory of a crystalline material of pancreatic origin, glucagon, has made available a model protein for fundamental biological and structural studies. This series of papers will present evidence leading to the elucidation of the complete amino acid sequence of glucagon. The present paper describes the quantitative amino acid analysis and other fundamental studies of primary importance to the sequence determination.

Experimental

Materials and Procedures.—The glucagon used throughout the structure work was a twice-recrystallized lot (#208-158B-292A) prepared from hog pancreas that was homogeneous according to end group analysis and zone electrophoresis on starch.[1] The high purity of the sample is also demonstrated by the analyses reported below which show good over-all recovery, a complete absence of isoleucine, proline and cystine, and, in general, good stoichiometric relationships of the amino acid residues.

Moisture content of the preparation was 10.9% as determined by drying for 24 hr. at 100° *in vacuo* over P_2O_5. The nitrogen content was 17.45% (Kjeldahl).

For the study of amino acid composition, accurately weighed samples of crystalline glucagon were hydrolyzed for 20 or 72 hr. in the absence of air at 107 to 109° in about 200 volumes of 5.7 *N* HCl redistilled three times in glass. The hydrolyzates were processed in a manner similar to that described by Smith and Stockell.[2] The hydrolyzates often contained traces of humin and had a faint blue-gray hue. The presence of trace metals in the crystalline preparation may have catalyzed the destruction of tryptophan since later crystalline preparations isolated in the presence of Versene produced light straw-colored hydrolyzates. Humin formation did not hamper the handling of the sample and probably did not affect the results.

Amino Acid Determination.—The comparatively rapid Levy[3] method, based on the paper chromatographic separation of the dinitrophenyl- (DNP-) amino acids, was employed initially in the work and was followed by the more rigorous Dowex 50 column chromatographic method of Moore and Stein.[4] The dinitrophenylation method furnished adequate preliminary data to support the structure work and, as will be reported, the two methods gave similar results.

The Dinitrophenylation Method.—Throughout this work dinitrophenylations were performed for 90 min. at 40°, *p*H 9.0, essentially following the Levy modification[3] of the method of Sanger.[5] Prior to the paper chromatographic separation of the DNP-amino acids, dinitrophenol was removed by sublimation according to the method described by Mills.[6] DNP-derivatives were identified by comparison with known DNP-amino acids.[7]

Only 20 hr. hydrolyzates were used with the Levy method since no DNP-peptides were observed. Recognizing that the correction factors published by Levy for the destruction of the amino acids in insulin may not be completely applicable to another protein handled under different conditions, new factors were determined for glucagon. This was accomplished by utilizing the insulin factors as a first approximation. From these data a synthetic amino acid mixture resembling glucagon was prepared, hydrolyzed and treated identically to a protein hydrolyzate. In this manner new destruction factors were calculated. This process was repeated, giving a more precise set of correction factors which were used in the calculation of the number of amino acid residues in glucagon. The correction factors are as follows: aspartic acid, 1.10; threonine, 1.22; serine, 1.32; glutamic acid, 1.20; glycine, 1.33; alanine, 1.28; valine, 1.20; methionine, 2.12; tyrosine, 1.60; phenylalanine, 1.20; histidine, 2.19; lysine, 0.70; and arginine, 1.38. Of interest is the finding that, in every instance, the factors are higher than those reported by Levy, averaging about 17% greater. Histidine and methionine particularly appear to be subject to greater losses than previously reported.[3]

Table I indicates the results of the preliminary amino acid analysis by the Levy method. With the relatively few analyses it is not surprising that the tyrosine and arginine values do not fall near whole-residue figures. Di-DNP-tyrosine analyses at best are quite variable,[3] and DNP-arginine, being water-soluble, is rather difficult to desalt completely prior to paper chromatography. Indeed, the water-soluble DNP-derivatives of histidine, lysine and arginine have generally proved to be difficult to handle. Ex-

TABLE I

AMINO ACID ANALYSIS OF GLUCAGON

Amino acid	Av. quantity found,[a] μM	Calcd. no. of amino acid residues[b]	No. of amino acid residues to nearest integer
Aspartic acid	0.34	4.0	4
Threonine	.24	2.8	3
Serine	.30	3.5	4[c]
Glutamic acid	.24	2.8	3
Glycine	.09	1.1	1
Alanine	.09	1.1	1
Valine	.08	0.9	1
Methionine	.06	.7	1
Leucine	.18	2.1	2
Tyrosine	.13	1.5	1 or 2
Phenylalanine	.17	2.0	2
Histidine	.11	1.3	1
Lysine	.08	0.9	1
Arginine	.22	2.6	2 or 3
Tryptophan[d]	.085	1.0	1
			28–30

[a] Average of 2 or 3 analyses with exception of single glycine determination. [b] On basis of 7 amino acids being present as single residues. (0.085 μM is average of 7.) [c] Since serine is subjected to approximately 10% destruction during acid hydrolysis (*cf.* Table II), 4 is a more reasonable figure than 3. [d] Chemical analysis.[8]

(1) A. Staub, L. Sinn and Otto K. Behrens, *J. Biol. Chem.*, **214**, 619 (1955).

(2) E. L. Smith and A. Stockell, *ibid.*, **207**, 501 (1954).

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

(4) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(5) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(6) G. L. Mills, *ibid.*, **50**, 707 (1952).

(7) Authentic DNP-amino acids were kindly supplied by Dr. Hans Neurath, Univ. of Washington, School of Medicine.

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

TABLE II

Gram amino acid residue per 100 g. protein

Amino acid	Time of hydrolysis: 20 hr. Hydrolyzate A		Hydrolyzate B		Av.	72 hr. Hydrolyzate A		Hydrolyzate B		Av.	Av. or extrap.*
Aspartic acid	13.3	13.1	12.8	13.3	13.1	12.8	12.8	12.7	12.2	12.6	13.3*
Threonine	8.17	8.16	8.18	8.28	8.20	7.74	(6.77)[a]	7.73	7.44	7.64	8.43*
Serine	8.63	8.93	8.75	8.57	8.72	6.75	6.64	6.89	6.75	6.76	9.62*
Glutamic acid	10.6	11.8	11.1	11.4	11.1	10.5	10.3	11.1	10.7	10.7	10.9
Glycine	1.82	1.61	1.45	1.51	1.60	1.93	1.44	1.40	1.46	1.56	1.58
Alanine	2.47	2.18	1.88	1.93	2.12	2.44	2.00	1.93	2.30	2.17	2.14
Valine	2.54	3.74	2.33	2.89	2.88	2.48	2.62	2.77	2.37	2.56	2.72
Methionine	3.33	3.99	2.89	3.84	3.51	3.22	2.83	3.20	3.31	3.14	3.32
Leucine	6.71	(8.05)	6.34	6.51	6.52	6.19	6.18	6.49	6.36	6.31	6.40
Tyrosine	9.09	8.99	(8.05)	9.51	9.20	(9.47)	8.34	7.95	8.52	8.27	9.59*
Phenylalanine	8.38	8.82	7.83	7.97	8.25	8.58	7.97	8.43	9.25	8.56	8.40
Histidine	3.85	3.28	3.38	3.51	3.51	3.25	2.98	3.21	3.49	3.22	3.37
Lysine	3.69	3.61	(2.73)	3.75	3.68	3.21	3.61	3.08	3.42	3.33	3.56
Ammonia ($-NH_2$)	3.02	2.65	2.75	2.96	2.85	3.80	3.78	3.22	3.37	3.54	2.62*
Arginine	8.75	(9.60)	8.62	8.87	8.75	7.88	8.40	8.43	8.82	8.38	8.54
Tryptophan[b]											5.20

[a] Values in parentheses not included in averages. [b] Average of 3 chemical analyses.[8]

traction from dilute acid solution into redistilled *n*-butanol provided a convenient method of preparing these derivatives for chromatography.

The values found for the other amino acids were reasonably close to whole residue units. The Levy method, used in this fashion, quickly and conveniently provided data on the amino acid composition of glucagon. These data provided an adequate working hypothesis during most of the structure determination.

Dowex 50 Method.—The elution curves in Fig. 1 illustrate the separation of amino acids achieved with the column chromatographic method of Moore and Stein.[4] The data of duplicate runs with two different hydrolyzates each at 20 and 72 hr. are given in Table II.

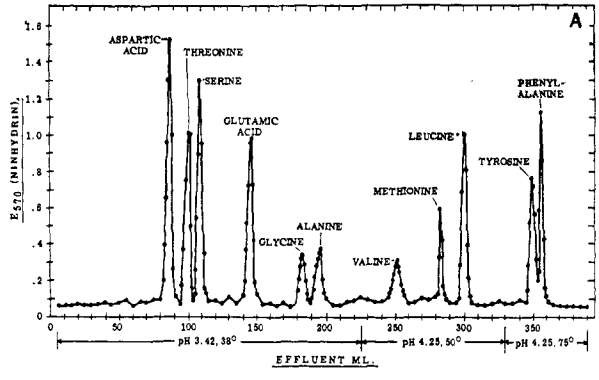

Fig. 1A.—Effluent curve of the neutral and acidic amino acids from a 72 hr. hydrolyzate of glucagon separated by chromatography on a 0.9 × 100 cm. column of Dowex 50-X8 resin. The quantity of hydrolyzate employed was 1.77 mg. The intensity of the color produced by reaction of the one ml. effluent fractions with ninhydrin was determined at 570 mμ in a Model B Beckman spectrophotometer. The elution curve obtained from these data is shown, with no corrections for base-line color or the various amino acid color yields.

In spite of the excellent repeatable separation of the amino acids, some variability in results was experienced. This was shown to be due to contamination with atmospheric ammonia. Because of these variations the data were subjected to the "t" test to determine whether or not the mean values found for 20 and 72 hr. differed significantly. It was found that the recoveries of aspartic acid, threonine, serine and tyrosine decreased with longer times of hydrolysis. Glutamic acid, methionine, histidine and arginine values appeared to decrease slightly; however, the difference between the 20 and 72 hr. values was not statistically significant.

The approximate percentage of destruction of these amino acids after 24 hr. of hydrolysis is: aspartic acid, 2%; threonine, 3.5%; serine, 11%; tyrosine, 5%. This rate of destruction is in reasonable accord with data published by Hirs, Stein and Moore,[9] Smith and associates,[2,10] and Rees[11] for other proteins and amino acid mixtures. Corrections for the losses of these acid-labile amino acids were calculated by extrapolation of the analytical data to zero time, assuming first-order kinetics for the decomposition (*cf.* 2, 9, 10). The method of calculation of Hirs, Stein and Moore[9] was employed.

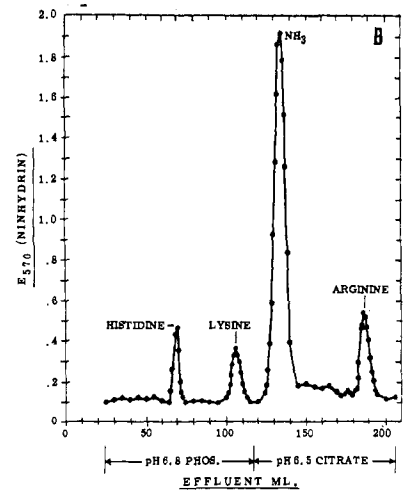

Fig. 1B.—Effluent curve of the basic amino acids from a 72 hr. hydrolyzate of glucagon separated by chromatography on a 0.9 × 15 cm. column of Dowex 50-X8 resin. The quantity of hydrolyzate analyzed was 1.77 mg. No corrections were applied for the base-line color or for the color yields of the various amino acids.

The amino acid composition and minimal molecular weight of glucagon calculated from these data are summarized in Table III.

(9) C. H. W. Hirs, W. H. Stein and S. Moore, *J. Biol. Chem.*, **211**, 941 (1954).

(10) E. L. Smith, A. Stockell and J. R. Kimmel, *ibid.*, **207**, 551 (1954).

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

TABLE III

AMINO ACID COMPOSITION AND MOLECULAR WEIGHT OF GLUCAGON

Amino acid	G. amino acid residue/100 g. protein	G. amino acid/100 g. protein	N as % total N	Minimal mol. wt.	Assumed no. of residues	Calcd. mol. wt.	Calcd. no. of residues/mol. wt. 3550
Aspartic acid	13.3	15.4	9.29	864	4	3460	4.11
Threonine	8.43	9.93	6.69	1200	3	3600	2.96
Serine	9.62	11.6	8.86	909	4	3640	3.91
Glutamic acid	10.9	12.4	6.22	1190	3	3570	2.98
Glycine	1.58	2.08	2.23	3610	1	3610	0.98
Alanine	2.14	2.68	2.42	3320	1	3320	1.07
Valine	2.72	3.22	2.21	3640	1	3640	0.97
Methionine	3.32	3.77	2.03	3960	1	3960	.90
Leucine	6.40	7.42	4.54	1770	2	3540	2.01
Tyrosine	9.59	10.6	4.70	1710	2	3420	2.08
Phenylalanine	8.40	9.42	4.58	1750	2	3500	2.03
Histidine	3.37	3.81	5.92	4070	1	4070	0.87
Lysine	3.56	4.06	4.46	3600	1	3600	.99
Ammonia	1.83[a,b]	1.94	9.16	876	4[a]	3500	4.05[a]
Arginine	8.54	9.52	17.6	1830	2	3660	1.94
Tryptophan	5.20	5.71	4.49	3580	1	3580	0.99
Totals	97.07	113.56	95.4		29	3550[c] 3600[d]	28.79

[a] Not included in totals or averages. [b] Direct chemical analysis.[11] [c] Average molecular weight from all residues except methionine and histidine, whose recoveries appeared to be low. [d] Average molecular weight for all residues.

The values for the stable amino acids represent averages of at least seven determinations. For most of the labile amino acids each point on the extrapolation curve was an average of four analyses. The amide NH_3 value listed was obtained by direct chemical analysis[11] and is an average of four determinations. Extrapolation of the chromatographic data yielded a significantly higher result. It is likely that this discrepancy may be attributed to the accumulation of NH_3 from air and the buffers. That this was indeed the case was verified by direct NH_3 analyses of the hydrolyzates prior to chromatography. The values obtained after correction for NH_3 from the destruction of serine and threonine (*cf.* 2, 9, 10, 11) were similar to those reported in Table III.

The average minimal molecular weight of 3550 was calculated on the basis that seven amino acids were present as single residues. This is in good agreement with the value of 3485 derived from the empirical formula of glucagon. Preliminary molecular weight determinations in 2 *M* guanidine solutions using the artificial boundary cell in the ultracentrifuge provided evidence for a molecular weight of approximately 4000.[12] The relative insolubility of glucagon and the small molecular size of the molecule render such physical studies difficult, and further determinations are planned.

An average yield of one mole of di-DNP-histidine was obtained per 3500 g. of glucagon. Direct chemical analysis[13] provided evidence for two free amino groups; these may be attributed to the N-terminal histidine and the ϵ-amino group of lysine. The small quantity of sulfur in the protein is completely accounted for by a single methionine residue.

The analytical data indicated a recovery of 97% of the weight of glucagon and 95% of the total nitrogen. These losses are reflected in the determined average molecular weight, 3550, which is about 2% higher than the value of 3485 calculated from the resulting empirical formula.

The data obtained from the two methods of amino acid analysis (*cf.* Tables I and III) is in good over-all agreement. In our hands, the data obtained from the Dowex 50 column method was more precise than that obtained from the dinitrophenylation technique. As previously discussed, the latter method utilizes inherently large correction factors. In addition, uncertainty exists in the analysis for certain DNP-amino acids. Advantages of the Levy method appear to be speed, ease of operation, use of a small sample, and the ability to simultaneously analyze a number of samples. The method was of particular value in this work as a rapid means of determining the approximate amino acid composition of glucagon, and later as a convenient method of analysis for certain of the smaller degradation peptides of glucagon.

N-Terminal Analysis.—Seven mg. (2 μmoles) of glucagon was dinitrophenylated for 90 min. according to the method of Levy.[3] Excess reagent was removed by extraction of the basic reaction mixture with ether. Upon acidification (*p*H 1.0) of the aqueous solution the yellow DNP-protein derivative precipitated. After washing with cold dilute acid, the precipitate was hydrolyzed in 5.7 *N* HCl for 24 hr. at 107–109°, and the excess HCl was removed *in vacuo* over KOH pellets. The yellow residue was redissolved in dilute acid and was extracted three times with ether, leaving essentially all of the yellow color in the aqueous phase. The water-soluble DNP-derivatives were subsequently extracted into *n*-butanol and were separated by the paper chromatographic system of Levy.[3] Two yellow DNP-amino acids were observed with properties indistinguishable from di-DNP-histidine and ϵ-DNP-lysine. A photograph of a chromatogram is given in Fig. 2. Calculations from the ultraviolet determination of the eluted spots indicated an average recovery from triplicate analyses of 0.49 mg. (1 μmole) of di-DNP-histidine per 3.5 mg. of glucagon. These data show that histidine is the N-terminal amino acid, and also corroborate that lysine is located within the chain.

C-Terminal End Group Analysis.—Two methods were used to examine the C-terminus of glucagon, hydrazinolysis[14] and carboxypeptidase treatment.

The hydrazinolysis of glucagon was carried out in a manner similar to that originally described by Akabori and coworkers.[14] Heating the dry protein with hydrazine caused a splitting of peptide bonds, with formation of the hydrazides of all amino acids except the C-terminal one. The hydrazides were removed, in this case by condensation with benzaldehyde to form dibenzals, and the free C-terminal amino acid was identified. Care must be exercised to exclude water from the reaction since hydrolysis of the peptide bonds may then occur. Glucagon was dried *in vacuo* for 24 hr. at 100° over P_2O_5. In a typical experiment 24.4 mg. (7 μM) of the dried preparation was dissolved in 3 ml. of fresh anhydrous hydrazine. The reaction mixture was quickly sealed under N_2 and was incubated at 125 ± 3° for 7.5 hr. The excess reagent was removed *in vacuo* over H_2SO_4. The dried, white residue was redissolved in 3 ml. of H_2O, and 0.3 ml. of benzaldehyde was added. The mixture was shaken for about 5 min., was centrifuged, and the light orange benzaldehyde phase was washed once with 1 ml. of water. The benzaldehyde layer was discarded, and the

(12) Value determined by E. O. Davisson and H. W. Fisher, Physicochemical Research Division, Lilly Research Laboratories.

(13) Determined using a time study with the method of D. D. Van Slyke, *J. Biol. Chem.*, **12**, 275 (1912).

(14) S. Akabori, K. Ohno and K. Narita, *Bull. Chem. Soc. Japan*, **25**, 214 (1952).

process was repeated 3 times with the combined aqueous phase and wash.

At the end of the treatment both the aqueous and organic phases were water-clear. The resulting aqueous phase, about 7 ml., was evaporated under a stream of nitrogen and finally was washed into a 1-ml. volumetric flask. Aliquots were removed for one-dimensional paper chromatography in *n*-butanol–acetic acid–water (4:1:1), and for two-dimensional chromatography according to the method of Redfield.[15]

These paper chromatographic systems provided fast, qualitative identification of amino acids and, in addition, each was adapted for use in quantitative amino acid analysis. The analytical method of Connell, Dixon and Hanes[16] was used in conjunction with the chromatographic system using the solvent butanol:acetic acid:water. In the two-dimensional system of Redfield the chromatograms were developed by use of 0.2% ninhydrin in acetone. The ninhydrin-reacted amino acid spots were excised and were eluted with 75% acetone. The solutions were subjected to spectrophotometric analysis at 570 mμ. In both methods standard recovery curves of all the amino acids in glucagon were employed for quantitative analysis. Both methods were found to be useful in the analysis of hydrolyzates of small peptides as well as in the determination of the hydrazinolysis product.

In both chromatographic systems one major hydrazinolysis product was observed, indistinguishable from threonine. A photograph of such a chromatogram after the method of Redfield is given in Fig. 3. Traces of methionine were also found in some experiments. In these analyses 0.7–0.9 mole of threonine was found per mole of glucagon. Since the hydrazinolysis reaction clearly released only threonine in good yield, this amino acid must be the C-terminal residue of glucagon.

Carboxypeptidase Method.—To confirm the hydrazinolysis result, and to obtain additional data concerning the amino acids near the C-terminal end, glucagon was incubated with carboxypeptidase; the amino acids which were liberated were determined at various intervals during the incubation. The method of amino acid analysis employed in this work was essentially that of Levy[3] and was first used in this manner by Harris, Li, Condliffe and Pon.[17]

Crystalline carboxypeptidase[18] was washed three times with cold distilled water. The concentration of the enzyme was determined prior to each experiment by removing an aliquot of the suspension, dissolving the enzyme in dilute alkali, and determining the absorption at 280 mμ. The crystalline enzyme (0.6 μM) was suspended in 1 ml. of 0.02 N $NaHCO_3$ and was dissolved by the dropwise addition of 0.1 N NaOH. The solution was immediately adjusted to *p*H 8.5 to 9 by the addition of 0.1 N HCl, and a 50-fold molar excess of diisopropylfluorophosphate (DFP) (0.1 M in isopropyl alcohol) was added. Upon the addition of DFP a sharp drop in *p*H occurred. The solution was quickly readjusted to *p*H 8.5 to 9 with 0.1 N NaOH, and was allowed to incubate at room temperature for 15 to 20 min.

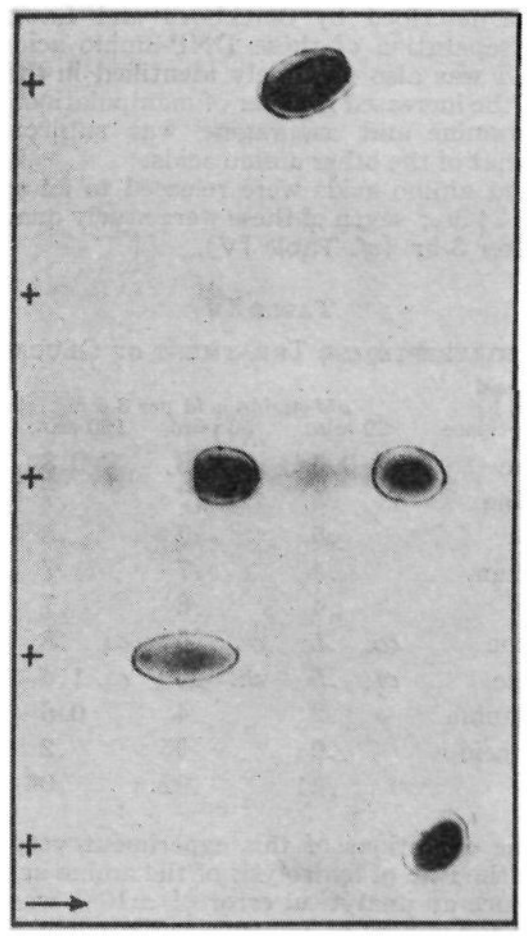

Fig. 2.—Photograph of the paper chromatographic separation of the DNP-derivatives resulting from the hydrolysis of DNP-glucagon. Reference lanes of authentic DNP-amino acids were run simultaneously. Spots were outlined in pencil for subsequent excision, elution and analysis.

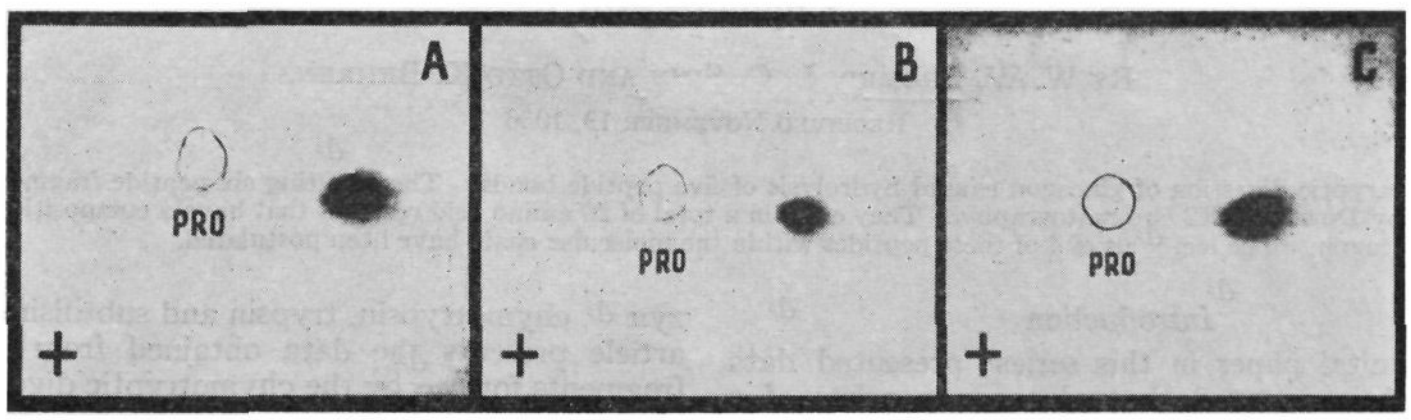

Fig. 3.—Photographs of the paper chromatographic analysis of the hydrazinolysis product of glucagon. Proline was added as a marker in all cases. Since ninhydrin reagent was used to visualize the amino acid spots, the faint yellow proline spot was outlined for purposes of identification: A, product of the hydrazinolysis of 0.04 μM of glucagon; B, threonine (0.03 μM); C, hydrazinolysis product plus 0.03 μM of threonine.

The enzyme solution was added to 14 mg. (4 μmoles) of crystalline glucagon dissolved in 5 ml. of dilute NaOH (*p*H 9.0). The enzyme:substrate molar ratio was approximately 1:65. The volume was adjusted to 7.5 ml., the solution was incubated at room temperature (34°), and appropriate aliquots were removed at 20, 50 and 180 min., as well as at 24 hr. The aliquots were pipetted directly into 15-ml. beakers containing HCl sufficient to adjust the *p*H to approximately 2.5. Those aliquots which were not immediately dinitrophenylated were kept frozen until they could be reacted. In each instance dinitrofluorobenzene was added prior to raising the *p*H to 9.0. The DNP-amino acids were separated by paper chromatography as discussed previously. Since DNP-asparagine and DNP-glutamine do not separate under these conditions this single spot was eluted and rerun

(15) R. R. Redfield, *Biochim. et Biophys. Acta*, **10**, 344 (1953).

(16) G. E. Connell, G. H. Dixon and C. S. Hanes, *Canad. J. Biochem. and Physiol.*, **33**, 416 (1955).

(17) J. I. Harris, C. H. Li, P. G. Condliffe and N. G. Pon, *J. Biol. Chem.*, **209**, 133 (1954).

(18) Kindly supplied by Dr. Hans Neurath, Univ. Washington School of Medicine.

on a system described by Blackburn and Lawther[19] that gives good separation of these DNP-amino acids. DNP-aspartic acid was also positively identified in this manner. Because of the increased number of manipulations, the analysis of glutamine and asparagine was subject to more error than that of the other amino acids.

At least 11 amino acids were released to an appreciable extent after 24 hr.; seven of these were nearly quantitatively liberated after 3 hr. (*cf.* Table IV).

TABLE IV

CARBOXYPEPTIDASE TREATMENT OF GLUCAGON

Amino acid liberated by carboxypeptidase	μM amino acid per 3.5 mg. glucagon 20 min.	50 min.	180 min.	24 hr.
Threonine	0.6	0.8	0.8	1.1
Methionine	.5	.7	.7	0.9
Valine	.5	.6	.8	.9
Tryptophan	.4	.7	.7	.9
Leucine	.4	.6	.7	.9
Asparagine	*ca.* .5	*ca.* .6	*ca.* .8	*ca.* 1.0
Glutamine	*ca.* .5	*ca.* .6	*ca.* 1.4	*ca.* 1.6
Phenylalanine	.2	.4	0.6	0.6
Aspartic acid	.02	.05	.2	.5
Alanine	.01	.02	.06	.3

Under the conditions of this experiment very little differential in the rate of hydrolysis of the amino acids was observed. Since an analytical error of $\pm 10\%$ was expected, no absolute assignment of sequence was possible. However, the data in Table IV indicate that threonine was probably liberated most rapidly by carboxypeptidase. These data, in conjunction with the clear-cut hydrazinolysis result showed that threonine is the carboxyl-terminal amino acid. The enzymatic release of methionine, tryptophan, valine and alanine is of considerable importance since these amino acids occur only once in the molecule. Obviously these amino acids can serve as markers for orientation of peptide fragments near the C-terminus of the molecule. Since a small amount of alanine and somewhat greater amounts of phenylalanine and aspartic acid were liberated, these amino acids are probably located at a position further removed from the C-terminus. Similarly, the gradual increase in the glutamine analysis to a value greater than one may be interpreted in terms of release of a second residue nearer the center of the molecule.

If optimum conditions had been found for the enzymatic reaction, additional sequential information could be obtained. Such experiments were planned, but before they had been conducted other data became available[20] that obviated the need for further carboxypeptidase experiments.

The evidence indicated that glucagon is a single chain polypeptide. The data make possible formulation of the following partial structure for glucagon.

$$\text{His(ser}_4\text{,glu,gly,thr}_2\text{,phe,asp}_2\text{,tyr}_2\text{,lys,leu,arg}_2\text{)ala(asp,phe,}\begin{matrix}\text{NH}_2\text{NH}_2\\ |\quad |\end{matrix}\text{glu}_2\text{,asp,leu,try,val,met)thr}$$

Acknowledgment.—The authors gratefully acknowledge the expert technical assistance of R. G. Scheib, C. T. Pugh and E. E. Logsdon.

(19) S. Blackburn and A. G. Lawther, *Biochem. J.*, **48**, 126 (1951).

(20) Papers II, III, IV and V in this series, THIS JOURNAL, **79**, 2798, 2801, 2805, 2807, (1957).

(21) The abbreviations for the amino acid residues used in these papers are those of E. Brand and J. T. Edsall, *Ann. Rev. Biochem.*, **16**, 224 (1947).

(22) Throughout this series of papers the arrangement of peptides is patterned after that suggested by F. Sanger, *Advances in Protein Chemistry*, **7**, 1 (1952).

INDIANAPOLIS 6, INDIANA

[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

The Amino Acid Sequence of Glucagon. II. The Hydrolysis of Glucagon with Chymotrypsin

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

RECEIVED NOVEMBER 19, 1956

Chymotryptic digestion of glucagon caused hydrolysis of five peptide bonds. The resulting six peptide fragments were isolated by Dowex 50-X2 chromatography. They contain a total of 29 amino acid residues that have a composition identical to glucagon. The locations of 4 of these peptides within the molecular chain have been postulated.

Introduction

The inital paper in this series[1] presented data which demonstrated that glucagon consists of a single chain of 29 amino acids with a molecular weight of 3485. Single residues of methionine, tryptophan, valine, alanine, lysine, glycine and histidine were found. Histidine and threonine were determined as the N- and C-terminal residues, respectively. Furthermore, incubation with carboxypeptidase showed that methionine, tryptophan, valine and a small quantity of alanine, were liberated from the C-terminal portion of the molecule. To determine the sequence of the 29 amino acids in this polypeptide, an enzymatic degradative approach[2] was followed using the proteolytic enzymes, chymotrypsin, trypsin and subtilisin. This article presents the data obtained from peptide fragments formed by the chymotryptic digestion of glucagon.

Experimental

Materials.—Twice-recrystallized glucagon (Lot 208-158B-292A) was employed for all experiments.

Crystalline chymotrypsin (Lot CD 521) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

Incubation of Glucagon with Chymotrypsin.—Glucagon (0.085%) was incubated with chymotrypsin (0.005%) at 37° in a dilute sodium acetate solution at *p*H 7.8 (enzyme: substrate molar ratio, about 1:120). Solution of the substrate was routinely accomplished by adjustment of the *p*H to 10 with 0.1 *N* NaOH. The reaction mixture was adjusted to *p*H 7.8 with 0.8 *N* HOAc and it remained essentially at this *p*H throughout the incubation.

In the preparative experiments the digests were acidified to *p*H 2.0, heated to 90° for 3 min., frozen and lyophilized.

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

(2) Consideration has been given to the possibility of enzyme-catalyzed rearrangements as first suggested by the work of M. Bergmann and O. K. Behrens, *J. Biol. Chem.*, **124**, 7 (1938); O. K. Behrens and M. Bergmann, *ibid.*, **129**, 587 (1939). Data obtained from a single enzyme should be interpreted with caution, and the examination of the degradation products from the action of more than one proteolytic enzyme is desirable.