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.

The rate, nature and extent of hydrolysis was investigated by dinitrophenylation[3] of aliquots of the reaction mixture removed at various time intervals. Samples that were not immediately reacted were treated as described above in the preparative experiments. The DNP-peptides were hydrolyzed for 20 hr. at 107° in 5.7 *N* HCl; the DNP-amino acids were separated by paper chromatography and were determined by the Levy[3] method. Corrections for the destruction of DNP-amino acids during acid hydrolysis were made using the data of Porter.[4]

This method not only provides an excellent means of determining the rate of the reaction, and thus the optimum time of digestion, but also provides evidence for the degree of splitting of the bonds, and gives information on the nature of the N-terminal residue of the newly formed peptides. The measurement of the DNP-amino acids provides reasonable indication of the quantity and the number of peptides actually liberated under the given hydrolytic conditions.

Dowex 50-X2 Separation of the Peptides.—The peptides in the enzymatic digests were resolved by column chromatography at room temperature with Dowex 50-X2, Lot 3328-12, 200 to 400 mesh, obtained from the Dow Chemical Company, Midland, Michigan. The resin and columns were prepared in a manner similar to that described by Moore and Stein.[5] For the purpose of packing the column, 0.1 *N* sodium citrate buffer at *p*H 3.0 was employed. The lyophilized digests were brought into solution using a minimum volume of this buffer, and were applied to the columns.

Columns 50 × 1.5 cm. were used in the isolation experiments, with a flow rate of about 7.5 ml. per hr. Fractions of 5 ml. were collected automatically, and 0.15 or 0.25-ml. aliquots were removed and analyzed by the quantitative ninhydrin method of Moore and Stein.[6] A gradient elution technique was employed using citrate buffers ranging (in 0.5 *p*H unit intervals) from *p*H 3 to 6.5, with the Na^+ concentration varying from 0.1 to 0.58 *N*. At appropriate intervals a higher *p*H, higher ionic strength buffer was applied, producing a reasonably smooth increase in both *p*H and ionic strength in the effluent. Volumes of about 300 ml. of buffer were used. All buffers contained 0.5% each of BRIJ 35, thiodiglycol and benzyl alcohol.

Preliminary Desalting of the Peptides.—After the peptides had been located in the effluent fractions by use of ninhydrin reagent, appropriate fractions were combined so as to include, in most instances, 95% of the peptide material. The combined fractions were swirled one hr. on a rotary shaker with 5 to 10 equivalents of Dowex 50-X4, Lot 707, 20 to 50 mesh, in the hydrogen form. The supernatant was removed by decanting and then drawing off the remaining solution with a small pipet. In most instances the supernatant gave no test with ninhydrin reagent and was discarded. In the event of a positive test, additional resin was added and the shaking was repeated. The resin beads were washed three times with 2 volumes of water, once with EtOH, and finally twice with water. They were then eluted by swirling with about 3 volumes of 5 *N* NH_4OH at 20 to 25° for 30 min. The beads were washed twice with NH_4OH and the combined eluates were evaporated *in vacuo* over P_2O_5 and concentrated H_2SO_4. The dry white residue was dissolved in dilute HCl (*p*H 3 to 4) and was washed into a volumetric flask. Small amounts of thymol and a broad spectrum antibiotic were added, and the solution was refrigerated to minimize growth of microörganisms. Sufficient salt and buffer-additives were removed by this method to permit direct paper chromatography of the peptides, or paper chromatographic separation of the peptide hydrolyzates. Pilot experiments indicated that significant quantities of peptide material were lost during the desalting procedure, depending upon the nature of the peptide. Despite such losses the desalting method was used routinely, since the presence of salts interferes markedly with the resolution of amino acids and proteins in paper chromatography. Paper chromatography was used extensively throughout the structure investigation because of the relative ease and speed of the method.

Analysis of Peptide Fragments.—An aliquot from each combined group of fractions from the Dowex 50 effluent was dinitrophenylated as previously described to ascertain the nature of the N-terminal residue and to provide additional evidence regarding the purity of the peptide itself. Certain peptides were subjected to 2-dimensional paper chromatography to provide additional information on homogeneity. Arbitrary corrections for destruction during acid hydrolysis were made for serine (10%), threonine (3.5%) and tyrosine (5%) (*cf.* 1). Yields of the peptides were based on both the DNP-end group and amino acid analyses, with the assumption that a given peptide occurred only once in the protein molecule. Hydrolyzates of the larger peptides were generally analyzed by the column method of Moore and Stein,[5] and/or by the dinitrophenylation method of Levy.[3] The hydrolyzates of the smaller peptides were analyzed by the two paper chromatographic methods reported in the preceding paper[1] in the section describing the C-terminal analysis.

Results

Rate of Hydrolysis.—A study of the rate of glucagon hydrolysis by chymotrypsin is presented in Fig. 1. If chymotrypsin follows the generally accepted specificity pattern, 5 bonds should be split adjacent to the 5 aromatic residues. The data presented in Fig. 1 are consistent with this expectation. Four different amino acids were found

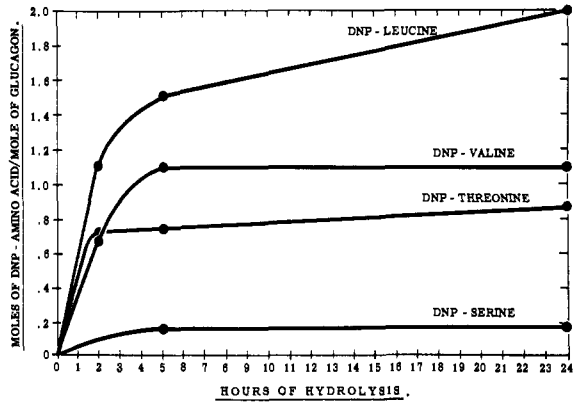


Fig. 1.—A study of the rate of the chymotryptic hydrolysis of the peptide bonds of glucagon, as followed by the dinitrophenylation method. Glucagon (0.085%) was digested with chymotrypsin (0.005%) at 37° in *p*H 7.8 buffer.

by the dinitrophenylation method as N-terminal residues of peptides newly formed by the chymotryptic rupture of peptide bonds Nearly quantitative splits were observed involving two leucine bonds, one valine bond and one threonine bond. In addition, a small yield of DNP-serine was obtained; data will be presented which indicate that the low recovery in the experiment is anomalous. In all likelihood a nearly quantitative split of the serine bond also was obtained. During the structure determination of corticotropin[7] similar difficulties with the dinitrophenylation method were experienced in determining serine as an N-terminal residue.

Separation of the Peptides.—In Fig. 2 is presented the Dowex 50-X2 effluent curve from the 8 hr. chymotrypsin digestion of 127 mg. (36.3 μmoles) of glucagon. Six major ninhydrin-positive peaks were detected, each of which represented a unique peptide fragment. Data on the analysis and recovery of each peptide are presented in Table I. No trace of peptide or amino acid impurities was

(3) A. L. Levy, *Nature*, **174**, 126 (1954).
(4) R. R. Porter, *Methods in Med. Res.*, **3**, 256 (1950).
(5) S. Moore and W. H. Stein *J. Biol. Chem.*, **192**, 663 (1951).
(6) S. Moore and W. H. Stein, *ibid.*, **211**, 907 (1954).
(7) A. L. Levy and C. H. Li, *ibid.*, **213**, 487 (1955).

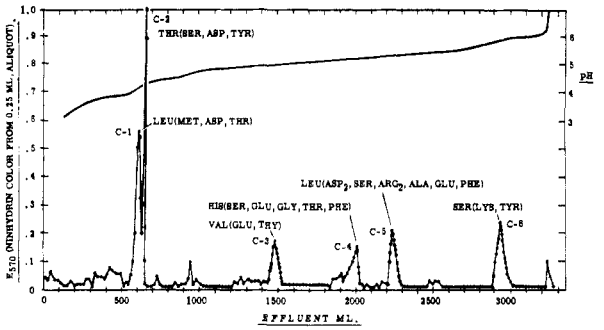


Fig. 2.—Separation of the peptides resulting from the 8 hr. chymotryptic hydrolysis of 127 mg. of glucagon. Chromatography was performed on a 1.5 × 50 cm. column of Dowex-X2 resin. The effluent was collected in 5-ml. fractions. The base-line variations were found to be caused by contamination with ammonia. A 0.2 *N* NaOH solution was used to clean the column, producing the final sharp rise in effluent *p*H.

detected in any of the 6 peptides by means of dinitrophenylation and quantitative amino acid analysis. These data, in addition to the behavior on the Dowex 50 column, clearly indicated that each of the peptides was essentially pure. The reasonable recovery of the peptides further attests that the isolated peptides are indeed fragments of glucagon. The yields of peptides C-3 and C-4 were in the same range as the other peptides, although their ninhydrin color yields were considerably lower. To rule out the possibility of overlooking peptides with low color yields, the fractions representing all small ninhydrin peaks were carefully investigated by paper chromatographic analysis after acid hydrolysis. No trace of other amino acids or peptides was found. It is likely that the small base-line variations exhibited in Fig. 2 may be attributed to ammonia contamination (*cf.* 1).

TABLE I

PEPTIDES FROM THE CHYMOTRYPTIC DIGESTION OF GLUCAGON

Peptide no.	Peptide compn. and anal.[a]	Yield, %
C-1	leu(met,asp,thr)	
	moles 1.0 0.8 1.2 1.0	40
C-2	thr(ser,asp,tyr)	
	moles 1.0 1.0 1.1 1.0	70
C-3	val(glu,try[b])	
	moles 1.3 0.6 1.0	60
C-4	his(ser,glu,gly,thr,phe)	
	moles 0.8 1.0 1.1 1.1 1.1 1.0	50
C-5	leu(asp_2,ser,arg_2,ala,glu,phe)	
	moles 0.9 1.9 1.0 1.8 1.0 1.0 0.8	50
C-6	ser(lys,tyr)	
	moles 0.9 1.3 1.0	50

[a] Hydrolyzates of the peptides were analyzed by the Moore and Stein[5] method, with the exceptions of C-3 and C-6 which were analyzed by paper chromatographic methods. [b] Chemical analysis.[8]

Twice as much C-6 peptide, containing N-terminal serine, was recovered as was expected from the rate study data. In view of this fact as well as the previously mentioned difficulty in the corticotropin structure work, it is likely that about the same degree of chymotryptic hydrolysis occurred in the case of the serine bond as was found with the other 4 bonds. If this hypothesis is correct the recovery of peptide C-6 was similar to that of the other peptides. The yields of the peptides are conservatively based on the assumption of 100% splitting, although the rate of hydrolysis data indicate that only 80 to 90% of the bonds were split after 8 hr. The shortest possible incubation time was chosen to minimize possible transpeptidation. The sum of the amino acid residues in all 6 peptides is 29, with a distribution of individual amino acids identical to that described for glucagon in the first paper of this series.[1] All evidence indicates that these peptides are fragments of glucagon produced by the specific hydrolytic action of chymotrypsin, and are not artifacts.

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

Discussion

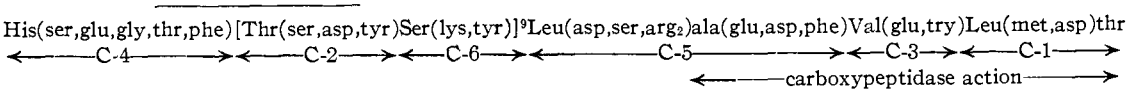
It is appropriate at this point to consider the probable location of these peptides in the glucagon chain. Since only one histidine residue occurs in the molecule and it has been located in the N-terminal position, peptide C-4, His(ser,glu,gly,thr,phe), must be the N-terminus of glucagon. Threonine was shown to be the C-terminal amino acid; but, since there are three threonine residues in the protein, each in a different chymotryptic peptide, the establishment of the C-terminal peptide would seem to be more difficult. However the C-4 threonine-containing peptide already has been designated as the N-terminal peptide. Similarly, the threonine residue in peptide C-2, thr(ser,asp,tyr), cannot be C-terminal in glucagon since it already has been shown to be the N-terminus of peptide C-2. By process of elimination, peptide C-1, Leu(met,asp, thr) must be the C-terminal peptide in glucagon. Other evidence also supports this conclusion; the single residue of methionine, that has been located near the C-terminus because of rapid release by carboxypeptidase, is found in this peptide. Similarly, the absence of an aromatic residue from C-1 is consistent with this interpretation; the usual specificity of chymotrypsin requires that the rupture involves the carboxyl group of an aromatic amino acid. Only the C-terminal fragment should be devoid of such a residue. Additionally, the location of aspartic acid and leucine in the C-terminal peptide do not in any way disagree with the data obtained from carboxypeptidase.

Carboxypeptidase also liberated tryptophan, valine and a small quantity of alanine. Since these three amino acids occur only once in glucagon, they should be found in peptides adjacent to peptide C-1, the C-terminal peptide. On this basis, peptide C-3, val(glu,try), containing both valine and tryptophan, may be placed adjacent to the C-terminal peptide, C-1. Seven of the 11 amino acids liberated by carboxypeptidase now have been placed in relative position near the C-terminus of the molecule. The four remaining amino acids include the single alanine residue, phenylalanine, glutamine and aspartic acid. Of the 3 unplaced peptides only C-5 contains the combination of aspartic acid, glutamic acid, phenylalanine and, of course, the single alanine residue. Hence it would appear that peptide C-5 is adjacent to peptide C-3 which

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



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[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.

(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).

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

(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).