nine, aspartic acid and glutamic acid, some of them as more than one residue. On treatment with carboxypeptidase, only leucine is released from this peptide. Assuming the prior removal of Glu·Phe from the C-terminus, this result is consistent with the C-terminal sequence: . . . . Pro·Leu·Glu·Phe.[8] Thus, it appears that chymotrypsin splits the C-terminal sequence between leucine and glutamic acid. The splitting of this bond and the failure to split at a second phenylalanine residue farther down the chain appear to be deviations from classical concepts[9] of chymotryptic specificity. However, the final answers to these and other[10] apparent anomalies of enzymatic activity must await the careful study of a wide range of synthetic peptides with highly purified enzymes.

Work is in progress to locate the exact position in corticotropin-A of the arginyltryptophan sequence. Present evidence indicates that it is not located near the termini.

Acknowledgment.—The authors wish to acknowledge the technical assistance of Mr. A. Gross.

(9) H. Neurath and G. W. Schwert, *Chem. Rev.*, **46**, 69 (1950).
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Studies on Pituitary Adrenocorticotropin. IX. Further Sequences Near the C-Terminus

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Short-term (2–4-hour) peptic hydrolyses of corticotropin-A give rise to only four peptide fragments showing significant ninhydrin-positive spots in paper chromatography. In these short-term hydrolyses there is little, if any, loss of physiological activity as measured by the Sayers test, although pepsin does cause destruction of activity if the action is prolonged.[1] Table I lists these peptic fragments together with the pertinent chromatographic, chemical and enzymatic data. Fragment No. 1, the slowest moving spot, is the only one from which physiological activity has been recovered[2] and contains all the amino acids found in corticotropin-A with the exception of leucine. This fragment apparently is the corticotropin-B of Brink, *et al.*[3] Fragment no. 2 is the tetrapeptide previously shown[4] to represent the last four amino acids at the carboxyl end of corticotropin-A. Since neither of the remaining two peptides contains serine, previously shown[5] to be the N-terminal amino acid of corticotropin-A, these sequences must also occur near the carboxyl end of the intact hormone. This conclusion is strengthened by the fact that all of the amino acids of the last three peptides of Table I are accounted for by two peptides isolated from the products of chymotryptic digestion of corticotropin-A and previously shown[6] to comprise a large section of the carboxyl end of the intact molecule. These peptides, together with pertinent data are shown in Table II.

The first peptide of Table II represents the last two amino acid positions in corticotropin-A and the second peptide extends from a point close to the center of the molecule out to the third position from the carboxyl end.

The problem of arranging the peptides of Table I in the structure of corticotropin-A was one of finding overlapping sequences. To this end, the long peptide of Table II was subjected to partial acid hydrolysis. The reaction was carried out by heating the peptide in 12 *N* hydrochloric acid at 37° for 72 hours. The resulting mixture was separated by paper chromatography and among the fragments were those listed in Table III. Fragment No. 1 ran very rapidly in both solvent systems and was clearly separated from all other ninhydrin-positive spots. Even without structural work this peptide clearly provided an overlap between fragments no. 2 and no. 3 of Table I and indicated an over-all arrangement of 1–3–4–2 in the fragments of corticotropin-A.

Fragment no. 2 of Table III was more difficult to separate and identify. After the second uni-dimensional chromatogram, the spot at Leu-/0.72 showed alanine, leucine and phenylalanine after complete acid hydrolysis, in the molar ratio: 2:1:1. In view of the known structures of the fragments of Table I and in view of the tentative over-all arrangement of these fragments, it was not possible to devise a logical single sequence containing two alanines, one leucine and one phenylalanine. Thus the Leu-/0.72 spot of Table III was adjudged to be a mixture of two dipeptides, each containing alanine. In terms of the tentative over-all arrangement, the most likely mixture was one of Leu·Ala and Ala·Phe. The former of these peptides was at hand[7] and its R_f values exactly fitted the data. By test it was found that Leu·Ala was not split appreciably by carboxypeptidase on 24-hour incubation. On the other hand, previous experience with a variety of natural and synthetic dipeptides, indicated that Ala·Phe would be completely split by carboxypeptidase in 24 hours. Accordingly, the Leu-/0.72 spot of Table III was subjected to 24-hour treatment with carboxypeptidase. A chromatogram of the product in the Partridge system showed alanine, phenylalanine and a spot above phenylalanine at $R_f = 0.72$ which, on complete acid hydrolysis, gave only alanine and leucine. Thus the second overlap (between fragments 4 and 3 of Table I) was confirmed.

Table IV summarizes all of the work published to date by this Laboratory on the C-terminal and of Corticotropin-A. The notation in this and in the other tables is that of Sanger.[8]

Acknowledgment.—The author wishes to ac-

(1) Under the enzymatic conditions used in this Laboratory (*cf.* Table I), losses of physiological activity become significant at about the sixtb hour and reach almost 100% at the twenty-fourth hour.
(2) W. F. White, W. L. Fierce and J. V. Lesh, *Proc. Soc. Exptl. Biol. Med.*, **78**, 616 (1951).
(3) N. G. Brink, *et al.*, THIS JOURNAL, **74**, 2120 (1952).
(4) W. F. White, *ibid.*, **75**, 4877 (1953).
(5) W. A. Landmann, M. P. Drake and W. F. Wbite, *ibid.*, **75**, 4370 (1953).
(6) W. F. White and W. A. Landmann, *ibid.*, **76**, 4193 (1954).
(7) Kindly furnished by Dr. Sidney W. Fox of Iowa State College, Ames, Iowa.
(8) "Advances in Protein Chemistry," Vol. VII, Academic Press, Inc., New York, N. Y., p. 5.

TABLE I

PEPTIDE FRAGMENTS SEPARATED FROM THE PRODUCTS OF SHORT-TERM (2–4-HOUR) PEPSIN DIGESTS OF CORTICOTROPIN-A[a]

Spot (peptide) no.	*Rf* values Part.[b]	*s*-But.–NH_3[c]	Amino acids on acid hydrolysis	N-Terminal determination	Order of release of amino acids by carboxypeptidase[d]	Sequence
1	0.05	Zero	Phe, Val, Met, Tyr, Pro, His, Glu, Asp, Ala, Arg, Lys, Gly, Ser (also Try by spectrophotometry)	DNFB:Ser	None	.....
2	.84	Tyr	Pro, Leu, Glu, Phe	Pos. isatin:Pro	Phe, Glu	Pro·Leu·Glu·Phe
3	.70	Arg^+	Ala, Glu, Phe	DNFB:Ala	Phe, Ala	Ala·Glu·Ala·Phe
4	.66	Glu-	Leu, Asp, Glu	DNFB:Asp	Leu	Asp·Glu·Leu

[a] Digestion carried out at 37° in 0.1*N* formic acid (*p*H 2.3) at corticotropin-A concentration of 5 mg./ml. with 1% pepsin crystalline Armour. [b] *n*-Butanol–water–acetic acid (4:5:1). [c] *s*-Butyl alcohol–ammonia (3:1). For details on the use of this system, *cf.*: J. F. Roland and A. M. Gross, *Anal. Chem.*, in press. Since this system is used over an extended period with a pad at the bottom of the sheet, R_f values are given in terms of the the nearest reference amino acid. [d] C-Terminal work was done by incubating the peptide at 37° in 0.1*N* ammonium acetate with approximately 1% crystalline carboxypeptidase in the presence of diisopropyl fluorophosphate. The reaction was followed at intervals over a 20-hour period. The sequences were deduced from the order of appearance of the amino acids.

TABLE II

TWO OF THE PEPTIDE FRAGMENTS SEPARATED FROM THE PRODUCTS OF CHYMOTRYPTIC DIGESTION OF CORTICOTROPIN-A[a]

Spot (peptide) no.	*Rf* values Part.	*s*-But.–NH_3	Amino acids on acid hydrolysis	N-Terminal determination	Order of release of amino acids by carboxypeptidase	Sequence
1	0.65	Arg+	Glu, Phe	DNFB:Glu	Little, if any, splitting	Glu·Phe
2	0.4–0.6	Zero	Lys, Val, Pro*, Gly, Ala*, Asp*, Glu*, Leu*, Phe	DNFB:Lys	Leu	Lys(Val, Pro*, Gly, Ala*, Asp*, Glu*, Phe)·Leu

[a] Quantitative results indicate that these amino acids are present as more than one residue.

TABLE III

TWO OF THE PEPTIDE FRAGMENTS SEPARATED FROM THE PRODUCTS OF PARTIAL ACID[a] HYDROLYSIS OF PEPTIDE NO. 2, TABLE II

Spot (peptide) no.	Part.	*Rf* values *s*-But.–NH_3	Amino acids on acid hydrolysis	Order of release of amino acids by carboxypeptidase	Sequence
1	0.89	1.4 × Phe	Ala, Pro, Leu, Phe	Leu	Ala·Phe·Pro·Leu
2	0.72	Leu	Ala, Leu	<10% split	Leu·Ala

[a] Hydrolyzed by incubating at 37° in concd. hydrochloric acid for 72 hours.

TABLE IV

SUMMARY OF RESULTS ON C-TERMINUS OF CORTICOTROPIN-A[a]

C-Terminal sequence (carboxypeptidase)													....Leu·Glu·Phe
Peptides from chymotryptic hydrolysis								Lys·(Val, Gly, Pro*, Ala*, Asp*, Glu*, Phe)·Leu				Glu·Phe	
Peptides from peptic hydrolysis						Asp·Glu·Leu			Ala·Glu·Ala·Phe			Pro·Leu·Glu·Phe	
Peptides from partial acid hydrolysis of large chymotryptic peptide									Leu·Ala			Ala·Phe·Pro·Leu	
C-Terminal sequence of corticotropin-A	....Lys·(Val, Pro, Gly, Ala, Glu, Asp)·Asp·Glu·Leu·Ala·Glu·Ala·Phe·Pro·Leu·Glu·Phe												
Bonds split by pepsin						↑			↑			↑	↑
Bonds split by chymotrypsin	↑											↑	
Probable termination point of corticotropin-B						↑							

[a] Quantitative values on these amino acids indicated that they were present as more than one residue.

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