Studies on Pituitary Adrenocorticotropin. VIII. Synthetic Confirmation of Three Dipeptides from Corticotropin-A

By W. F. White and W. A. Landmann

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By means of paper chromatography we have isolated three dipeptides from the products of chymotryptic digestion of corticotropin-A. All three of these dipeptides have been compared with the corresponding synthetic compounds and in each case the properties of the natural and synthetic materials were identical.

In the enzymatic treatment of corticotropin-A, 1% α-chymotrypsin[1] was used at 37° for 24 hours in 0.1*N* ammonium acetate buffer. After removal of the buffer by lyophilization, the residue was subjected to two-dimensional paper chromatography to locate the hydrolytic fragments. In the first dimension, the Partridge system[2] was used over a 24-hour period and in the second dimension, the system *s*-butyl alcohol–ammonia (3:1)[3] was employed over a 48-hour period with the solvent being allowed to run into a paper wad at the bottom of the sheet. Ninhydrin revealed several diffuse, difficultly separable spots near the origin and three "tight," well-separated spots further down the chromatogram. Samples of the latter three substances were obtained by successive uni-dimensional chromatograms in the same solvent systems.

The three peptides were examined by the methods developed by Sanger, yielding the data summarized in Table I. The structures of the first and third peptides were clear from the data. The second spot was not free arginine as shown by the R_f values and from the fact that it migrated more slowly toward the cathode than arginine in paper electrophoresis at *p*H 7. However, since tryptophan had been detected in corticotropin-A, the possibility existed that the unknown spot was a dipeptide of arginine and tryptophan. To test this possibility, the action of carboxypeptidase was tried.[4] On digestion of material from spot 2 at 37° for 20 hours at *p*H 7.8, the original spot disappeared in favor of two spots at the proper location for tryptophan and arginine. Combined with the results of the DNFB test, this indicated the structure Arg·Try.

Table I

Spot No.	R_f Partridge	R_f *s*-But.:NH_3	Amino acids on complete acid hydrolysis	N-Terminal amino acid by DNFB	Structure
1	0.65	Arg+	Glu,Phe	Glu	Glu·Phe
2	.46	Val	Arg	Arg	Arg·Try
3	.42	Pro	Ser,Tyr	Ser	Ser·Tyr

In order to confirm the tentative structures indicated in the last column of Table I, the properties of the natural dipeptides were compared with those of synthetic dipeptides obtained through the courtesy of Dr. Klaus Hofmann, Biochemistry Department, University of Pittsburgh, School of Medicine.[5]

Of the three synthetic products, the glutamylphenylalanine and the seryltyrosine gave single ninhydrin spots in paper chromatography and the R_f values exactly duplicated those of the corresponding natural peptides. In addition, acid hydrolysis and DNFB treatment gave the same results as are shown in Table I for the corresponding natural compounds. On the other hand, the arginyltryptophan showed two spots with ninhydrin after chromatography in the Partridge system. The stronger spot corresponded to that of the natural arginyltryptophan, while the weaker had an R_f value of 0.57. Several milligrams of the arginyltryptophan preparation was fractionated in the Partridge system on Whatman #3 paper, the two components being eluted from their respective positions after developing a marker strip with ninhydrin. The properties of the R_f = 0.46 component duplicated those of the natural arginyltryptophan.

As a final test, the natural and synthetic peptides were compared by incubation with carboxypeptidase. The enzyme was treated with diisopropyl fluorophosphate and *d*-leucyl-l-tyrosine was carried along with the samples as a control substance. Digestion was carried out at 37° in *p*H 7.7 0.1*N* ammonium acetate buffer for 20 hours. Under these conditions, the control peptide and both the natural and the purified synthetic arginyltryptophan were completely split into their constituent amino acids. On the other hand, little or no splitting was observed with the glutamylphenylalanine peptides. Lastly, the seryltyrosine peptides were split to an extent estimated at about 25%.[6] In all cases, the results with the natural and synthetic peptides were the same.

In a previous communication[7] the sequence Ser·Tyr· was shown to be located at the N-terminus of corticotropin-A. Present evidence indicates that the glutamylphenylalanine dipeptide described above represents the two amino acids previously shown to be located at the C-terminus.[8] The evidence for this is as follows: The third amino acid from the carboxyl end of corticotropin-A is known to be leucine.[8] Only one fragment containing leucine has been isolated from the products of chymotryptic digestion of corticotropin-A. This peptide forms a long, diffuse spot in the Partridge system extending from 0.35 to 0.60 and is immobile in the *s*-butyl alcohol–ammonia system. On complete acid hydrolysis, it shows, in addition to leucine: lysine, valine, proline, glycine, phenylalanine, ala-

(1) Three times crystallized Armour #381-092.

(2) *n*-Butanol-water-acetic acid (4:5:1).

(3) Since this system is used in such a way that the position of the solvent front cannot be determined, R_f values are given in terms of the nearest amino acid. For details on the use of this solvent, *cf.* J. F. Roland and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954).

(4) A previous attempt to split this peptide by means of trypsin was unsuccessful. After incubation at 37° for 3 hours with 1% crystalline trypsin, at *p*H 7.75 in 0.1*N* ammonium acetate buffer, paper chromatography showed no evidence of change.

(5) The preparation and properties of these synthetic dipeptides will be described elsewhere. In each case the starting materials were the *l*-isomers of the amino acids and the method was known not to cause racemization.

(6) In a personal communication, Dr. J. Ieuan Harris, Department of Biochemistry, University of California, has reported that he has synthesized seryltyrosine and that his preparation is also split by carboxypeptidase.

(7) W. A. Landmann, M. P. Drake and W. F. White, This Journal, **75**, 4370 (1953).

(8) W. F. White, *ibid.*, **75**, 4877 (1953).

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).
(10) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

THE ARMOUR LABORATORIES
CHICAGO, ILLINOIS

Studies on Pituitary Adrenocorticotropin. IX. Further Sequences Near the C-Terminus

BY W. F. WHITE

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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 sixth 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. White, *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.