[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

Acid Hydrolysis and the Amino Acid Composition of Sheep Pituitary Adrenocorticotropic Hormone

BY CHOH HAO LI

The percentage of amino acids in the adrenocorticotropic hormone isolated from whole sheep pituitaries has been determined by microbiological and chemical techniques. 98.8% of the protein nitrogen has been accounted for by its content of known amino acids and the amide nitrogen. The molecular weight has been estimated to be 22,600 by calculations based on the content of five amino acids. The hydrolysis of the hormone in 1 *M* HCl at 100° has been studied. It was found that some hormonal activity was retained after the protein had been hydrolyzed to the extent of 90%. It was also demonstrated that the ACTH peptide mixture obtained by pepsin digest of the protein hormone preserves its activity after further hydrolysis in 1 *M* HCl.

It has previously been shown[1] that adrenocorticotropic hormone (ACTH) retains its activity after partial hydrolysis in 6 *M* HCl at 37°. More recently we were able to demonstrate that the adrenal-stimulating activity of the hormone is enhanced by acid–heat treatment.[2] Further studies on the hydrolysis of ACTH with acid, together with its amino acid composition are herein reported.

Experimental

Adrenocorticotropic hormone was isolated from fresh sheep pituitary glands by the procedure previously described.[3] The hormone was assayed in hypophysectomized male rats by the technique of Sayers, *et al.*[4] It was found[4,5] that a plot of the dose in logarithm scale against the depletion of adrenal ascorbic acid gave a straight line relationship. From this standard curve, the potency of an unknown preparation can be estimated from the amount of adrenal ascorbic acid depletion.

The purity of ACTH preparations has been examined by electrophoresis in *p*H 3.0 NaCl–HCl buffer of 0.1 ionic strength using the Tiselius technique.[6] In each case, a single boundary was observed indicating that the preparations were homogeneous with respect to the electrochemical characteristic. One of the preparations was also subjected to ultracentrifuge and diffusion studies.[7] It was found that the preparation behaved as a homogeneous protein with the following physical constants: $S_{20} = 1.97S$ and $D_{20} = 9.0 \times 10^{-7}$.

Hydrochloric acid was used throughout in the present investigation. The microbiological assay procedures were employed for the determination of amino acids and were carried out in the Shankman Laboratories. Lysine, tyrosine, arginine and histidine were also determined chemically. The colorimetric methods of Kibrick[8] and of Lugg[9] were employed for the determination of lysine and tyrosine, respectively. Arginine and histidine were estimated by the procedure of Macpherson.[10]

The free amino nitrogen was determined in the manometric Van Slyke apparatus[11] and the free amino acid nitrogen by the gasometric ninhydrin reaction.[12]

The amide nitrogen was determined by the method previously described,[13] and the total nitrogen by the Kjeldahl procedure. One-way paper chromatography was carried out by the procedure of Consden, Gordon and Martin[14]; Whatman No. 1 paper was used. Butanol–acetic acid (10%)/H_2O was employed as the solvent and was prepared as suggested by Partridge.[15] The solutions (0.1-cc. samples) to be chromatographed were evaporated to dryness in a vacuum desiccator and the dried material was then dissolved in 0.02 cc. of water and applied in the paper.

The degree of hydrolysis of the hormone was computed from its solubility in 5% trichloroacetic acid solution. As the native protein is only soluble to variable extent (2–5%) in the trichloroacetic acid, the increment of the solubility after the treatment with acid is taken as indicating the percentage of hydrolysis.

Results

Amide Nitrogen.—From 4 different preparations of the hormone, the amide-N content in the HCl hydrolysates averaged 7.9% of the total nitrogen. As also shown in Table I, 78.0% of the total N was derived from the free amino N.

TABLE I

AMIDE-N AND AMINO-N OF HCl HYDROLYSATES[a] OF ADRENOCORTICOTROPIC HORMONE

Values are given as per cent. of total N

Preparation	NH_2-N, %	Amide-N, %
I (1)[b]	79.0	7.3
II (1)	80.4	6.7
III (3)	80.5	9.7
IV (2)	72.0	7.9
Mean	78.0	7.9

[a] Twenty mg. of the hormone was hydrolyzed in a sealed tube with 1 cc. of 20% HCl at 100° for 24 hours. [b] Number of determinations in parentheses.

Amino Acid Composition.—Table II summarizes the amino acid composition of the adrenocorticotropic hormone. It may be noted that the values obtained by the chemical and microbiological methods are in good agreement; the values for cystine and methionine are taken from an earlier chemical analysis[16] and the sulfur content in the protein is totally accounted for by these two amino acids. The alanine content has not been analyzed; variable results were obtained with the tryptophan determination. In a number of determinations, the value of tryptophan was found to vary from 0.6 to 1.0%. We are inclined to believe that the high value represents the true tryptophan content in the hormone. For reasons of uncertainty, however, the tryptophan content is not included in Table II.

Table III presents the calculated minimum molecular weight of the hormone, based upon its content in histidine, phenylalanine, tyrosine, threonine and methionine. The value 22,600 is not greatly

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(2) Li, THIS JOURNAL, **72**, 2815 (1950).

(3) Li, Evans and Simpson, *J. Biol. Chem.*, **149**, 413 (1943).

(4) Sayers, Sayers and Woodbury, *Endocrinology*, **42**, 379 (1948).

(5) Greenspan, Li and Evans, *ibid.*, **46**, 261 (1950).

(6) Tiselius, *Trans. Faraday Soc.*, **33**, 542 (1937).

(7) The author is indebted to Dr. Kai O. Pedersen of Uppsala for these experiments, the details of which will be reported elsewhere.

(8) Kibrick, *Arch. Biochem.*, **20**, 22 (1949).

(9) Lugg, *Biochem. J.*, **32**, 775 (1937).

(10) Macpherson, *ibid.*, **40**, 470 (1940). I am indebted to Dr. Macpherson for these determinations.

(11) Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).

(12) Hamilton and Van Slyke, *ibid.*, **150**, 231 (1943).

(13) Li, *ibid.*, **178**, 459 (1949).

(14) Consden, Gordon and Martin, *Biochem. J.*, **38**, 224 (1944).

(15) Partridge, *ibid.*, **42**, 238 (1948).

(16) Li, *Federation Proc.*, **5**, no. 1, March 1946.

TABLE II

AMINO ACID ANALYSIS OF ADRENOCORTICOTROPIC HORMONE

Amino acid	G. per 100 g. protein	Methods
Arginine	8.7	Microbiological[a]
	8.5	Chemical[b]
Aspartic acid	6.7	Microbiological[a]
Cystine	7.2	Chemical[c]
Glutamic acid	15.6	Microbiological[a]
Glycine	8.0	Microbiological[a]
Histidine	1.3	Microbiological[a]
	1.4	Chemical[b]
Isoleucine	3.1	Microbiological[a]
Leucine	7.8	Microbiological[a]
Lysine	5.0	Microbiological[a]
	4.8	Chemical[d]
Methionine	1.4	Microbiological[a]
	1.9	Chemical[e]
Phenylalanine	4.0	Microbiological[a]
Proline	8.2	Microbiological[a]
Serine	6.0	Microbiological[a]
Threonine	3.2	Microbiological[a]
Tyrosine	2.4	Microbiological[a]
	2.5	Chemical[e]
Valine	3.4	Microbiological[a]

[a] Determined by Shankman Laboratories. [b] Determined by Macpherson.[8] [c] Taken from Li.[12] [d] The method of Kibrick[6] used. [e] The method of Lugg[7] was used.

TABLE III

MINIMUM MOLECULAR WEIGHT OF ADRENOCORTICOTROPIC HORMONE

Amino acids	G. per 100 g. protein	Minimum molecular weight	Av. no. of residues	Calculated molecular weight
Histidine	1.3	11,940	2	23,880
Methionine	1.9	7,860	3	23,580
Threonine	3.2	3,720	6	22,320
Tyrosine	2.4	7,550	3	22,650
Phenylalanine	4.0	4,130	5	20,650
			Mean	22,616

TABLE IV

COMPOSITION OF ACTH PROTEIN

(Molecular Weight 22,600)

Constituent	G. per 100 g. protein	N as % of protein N	Estimated no. of residues
N	15.6		
S	2.3		
Amide-N	1.2	7.9	
Arginine	8.7	17.9	11
Aspartic acid	6.7	4.5	11
Cystine	7.2	5.4	7
Glutamic acid	15.6	9.5	24
Glycine	8.0	9.6	24
Histidine	1.3	2.3	2
Isoleucine	3.1	2.1	5
Leucine	7.8	5.3	13
Lysine	5.0	6.1	8
Methionine	1.9	8.4	3
Phenylalanine	4.0	2.2	5
Proline	8.2	6.4	16
Serine	6.0	5.1	13
Threonine	3.2	2.4	6
Tyrosine	2.4	1.2	3
Valine	3.4	2.6	7
Total		**98.9**	158

different from that determined by molecular kinetic studies.[17]

The protein nitrogen distribution and the estimated number of amino acids residues per mole (22,600) are shown in Table IV. It may be seen that the known amino acids together with the amide-N account for 98.9% of the protein nitrogen. There are 158 amino acid residues excluding the values for alanine and tryptophan.

Hydrolysis in 1 *M* HCl.—When a 1% solution of the ACTH protein in 1 *M* HCl was kept in a boiling water-bath (100°) for 120 minutes, it was found that the protein became completely soluble in 5% trichloroacetic acid and its adrenal-stimulating activity was destroyed. The free amino nitrogen increased from 0.8 to 5.1% indicating that a definite hydrolysis of the peptide chains occurred. On the other hand, only a small amount of free amino acid was liberated by the treatment. Paper chromatographs of the whole solution after heat treatment at various time intervals are shown in Fig. 1. It is

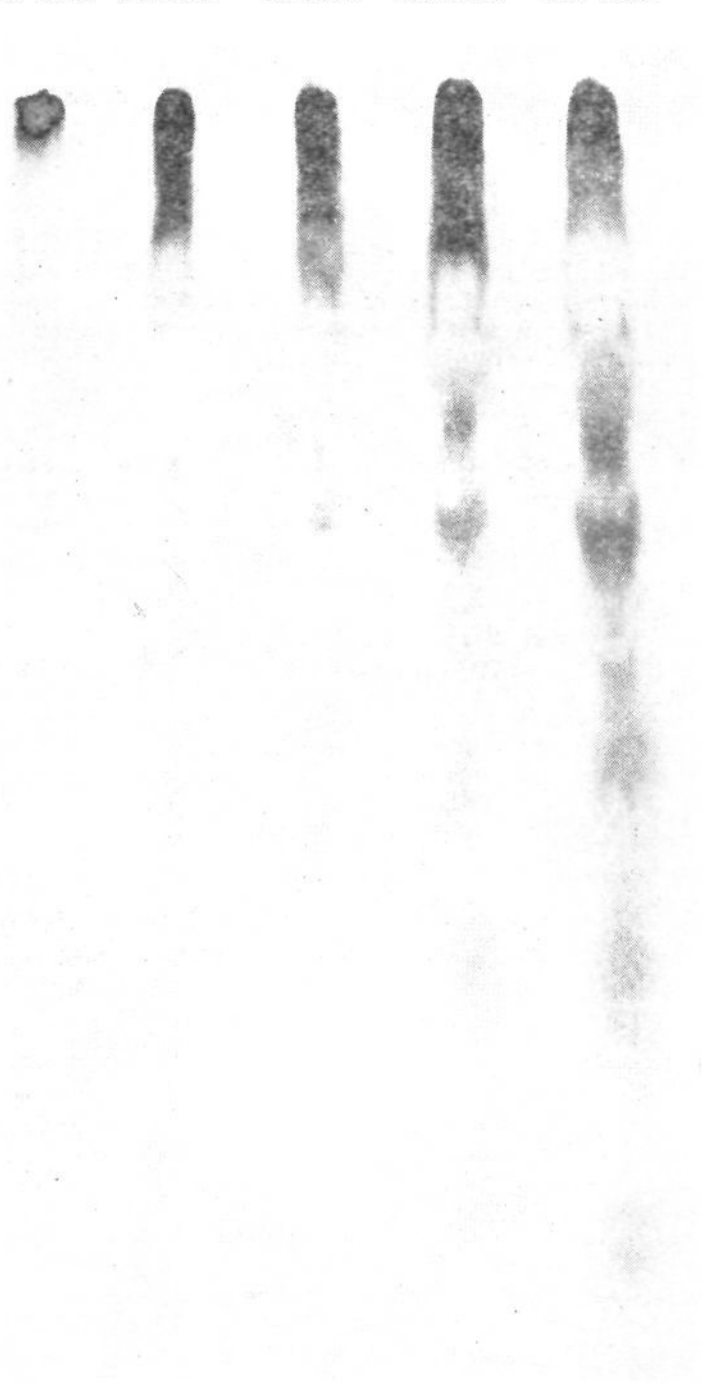

Fig. 1.—Paper chromatography of the hydrolysates of the ACTH protein in 1 *M* HCl at 100°; solvent, butanol–10% acetic acid/H_2O.

(17) Burtner, THIS JOURNAL, **65**, 1238 (1943)

evident that the number of ninhydrin-positive areas and the intensity increase as the degree of hydrolysis increases.

Results of a typical experiment are summarized in Table V; in the same table, the bioassay of the 5% trichloroacetic acid soluble fraction are also presented. It is to be noted that when the protein hormone is hydrolyzed to an extent of 52% no loss of activity is observed. Further hydrolysis causes a decrease of the hormone potency, but some activity is still retained up to 90% of hydrolysis. It must be emphasized that the assay data of Tables V and VI are only roughly quantitative. Statistically, a significant difference is found only between the completely hydrolyzed samples and any of the others.

TABLE V

ACID HYDROLYSIS[a] OF ADRENOCORTICOTROPIC HORMONE

Time, min.	Degree of hydrolysis, %	NH_2–N,[c] %	Free amino acid–N,[c] %	Depletion of ascorbic acid at 1 microgram nitrogen level,[b] mg./100 g. adrenal	ACTH activ. equivalent, microgram
0	0	0.8	0.0	−127, −116, −131	6.0
10	27	1.6	.2	−134, −138, −125, −145, −142, −167	10.5
25	52	2.5	.2	−195, −117, −149, −132, −111, −146	10.5
40	72	2.7	.3	−129, − 99, − 57, −177, − 67, − 84, − 75	2.5
80	91	4.1	.6	−124, − 82, − 91	2.6
120	100	5.1	1.2	+ 35, − 9, + 44, + 27	0

[a] 1% solution of ACTH protein in 1 *M* HCl at 100°. [b] One microgram nitrogen of the 5% TCA soluble fraction per 100 g. body weight of hypophysectomized rats was used. [c] Values are given in per cent. of solid.

TABLE VI

ACID HYDROLYSIS[a] OF ACTH ACTIVE PEPTIDE MIXTURE

Time, min.	Depletion of ascorbic acid at 5 microgram level, mg./100 g. adrenal	ACTH activity equivalent microgram
0	− 97, −139, −170, −150, − 97, −139	8.0
15	− 72, −164, − 96, −170, −224	12.5
30	−100, − 96, −138, −117	4.2
60	+ 32, + 1, − 5	0

[a] 1% solution of ACTH peptide in 1 *M* HCl at 100°.

This remarkable property of the ACTH protein is also exhibited by the active peptide mixture[1,19] prepared from a pepsin digest. Using the same conditions of treatment, it was found that the adrenal-stimulating activity of the ACTH peptide mixture was retained after the peptide solution (1% in 1 *M* HCl) was kept at 100° for 15 minutes, while the free amino-N as determined by the Van Slyke procedure changes from 1.4 to 2.2%. As shown in Table VI, some loss of the activity occurs if the treatment extends to 30 minutes; in one hour the adrenal ascorbic acid depletion activity of the ACTH is completely abolished.

It has previously been reported[3] that the ACTH protein contains a trace of posterior pituitary hormones. Preliminary studies[19] indicate that the trace contaminants were abolished by 1 *M* acid–heat treatment.

Discussion

It is evident from Table IV that the protein nitrogen in the hormone is accounted for by the known amino acids. Although we do not have the data for alanine, qualitative analysis indicates that it is present. In fact, we have obtained good evidence[20] that the hormone has only one terminal amino end-group and that this end-group is alanine. As reported in an earlier communication,[3] the protein hormone contains no phosphorus, carbohydrate and –SH groups.

One of the unusual properties of the ACTH protein is resistance to coagulation by heat. In contrast to other proteins, the hormone solution remains clear at *p*H 7.0 after being kept in a boiling water-bath for many hours. The data presented herein show that the hormone molecule possesses an even more remarkable nature in that the hormonal activity is retained after partial hydrolysis with acid at 100°.

Previous studies[1,18] have clearly demonstrated that the ACTH activity resides in the peptide residue of the protein hormone. The results recorded in Table V furnish additional evidence that a small fragment is responsible for the adrenal-stimulating activity. The fact that the ACTH peptide mixture obtained by pepsin digest can be further hydrolyzed by 1 *M* HCl and still retain biologic potency indicates either that the ACTH active peptide in the mixture is resistant to acid hydrolysis or that the active peptide is broken into a smaller size and still preserves its activity.

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(18) Li and Pedersen, *Arkiv. Kemi*, 1, 533 (1950).

(19) Hungerford and Reinhardt, unpublished data on antidiuretic activity in hydrated rats.

(20) Porter and Li, unpublished data.