[CONTRIBUTION FROM THE CHEMICAL LABORATORY, IOWA STATE COLLEGE]

Sequential and Amino Acid Residue Compositions of Adrenocorticotropic Hormone Preparations of Various Levels of Activity[1]

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A subtractive microbiological phenyl isothiocyanate and dioxane–HCl sequential analysis of peptides has been shown to be applicable to natural peptides. The particular technique used is also applicable in the presence of chemically functional side chains of amino acid residues. Analyses for a number of ACTH preparations of varying levels of activity have been presented and compared with data from other laboratories. L-Serine has been shown to be N-terminal in Corticotropin A, with L-tyrosine as a penultimate residue. Other aspects of structure of ACTH have been discussed. Current evaluations of advantages and disadvantages of the methods used have been presented.

This paper presents data on the application of a quantitative subtractive microbiological sequence technique[2] to ACTH preparations of various levels of biological activity. The resultant data permit comparisons to be made with published figures for such and other preparations, and also permit consideration of the meaning of these results to an understanding of the structure of the hormone.

Experimental

Materials.—The ACTH preparations were furnished by Dr. W. F. White of Armour and Co.

Analytical Method.—The chemical method was virtually the same as described earlier.[2] In a typical determination of residues in the position next to the aminoid terminus (AT_2), 1.00 ml. of aqueous sample containing 500 γ of material was treated with 1.50 ml. of pyridine containing 0.01 ml. of phenyl isothiocyanate (PTC). This was maintained at *p*H 7.5–8.0 with a little added brom thymol blue by occasional addition of 0.05 *N* sodium hydroxide solution. The solution was incubated at 37° for 6–12 hr. (until no further drop in *p*H was observed). After drying in a vacuum desiccator over sodium hydroxide and sulfuric acid, the residue was treated with 2.0 ml. of dioxane saturated with hydrogen chloride, in a small desiccator for 6–12 hr. The liquid was evaporated in a vacuum desiccator over sodium hydroxide and sulfuric acid, and the residue carried through the same PTC treatment again. To the residue from this operation was added 5.0 ml. of 6 *N* hydrochloric acid, and the mixture was autoclaved at 15 lb. pressure for 16 hr. The solution was then evaporated over steam and made up in a volumetric flask with sufficient sodium hydroxide to bring the *p*H to 6.8–7.0, and then assayed. For estimating terminal residues only (AT_1) the dioxane–HCl treatment was omitted; in AT_3 there were two dioxane–HCl and one aqueous HCl treatments, etc. AT_0, AT_1, AT_2, AT_3, AT_4 samples were each processed individually, although all of the amino acids estimated were assayed on each such preparation.

Assay Organisms.—The organisms employed for microbiological assay included *Lactobacillus arabinosus* 17-5 (a), *Lactobacillus brevis* (b), *Streptococcus faecalis* (f), and *Leuconostoc mesenteroides* (m). The corresponding ATCC numbers were 8014, 8287, 8043 (9790 in Table IV) and 8042. In the tables, the organisms used are indicated by the appropriate letters in parentheses, as are the media.

Media.—Eleven media were employed. These were as follows: p, same as medium of Kuiken, Lyman and Hale[3]; q, same as medium of Kuiken, *et al.*,[4] except that in each liter of medium there were included 40 g. of sodium acetate, 470 mg. of L-glutamic acid, 300 mg. of L-lysine hydrochloride, 800 mg. of DL-tryptophan, 400 mg. of L-tyrosine, 2000 γ of thiamine hydrochloride, 400 γ of pyridoxine hydrochloride, 400 γ of calcium pantothenate, 10 γ of biotin and 400 γ of *p*-aminobenzoic acid, instead of the corresponding amounts or forms listed. In addition were included 400 mg. of DL-norleucine, 400 mg. of glycine, 2 γ of folic acid, and no tomato eluate preparation. Media r–w were each the same as q except for the following modifications: r, 15 g. of sodium acetate[4]; s, acetate replaced entirely by 15 g. of succinic acid[5]; t, 30 g. of glucose and 10 g. of arabinose[6]; u, 20 g. of glucose and 20 g. of arabinose; v, 30 g. of glucose, 10 g. of arabinose and 400 mg. of L-glutamic acid; w, 400 mg. of DL-aspartic acid w, 400 mg. of DL-aspartic acid[7]; x, Medium VI of Steele, *et al.*,[8] with, however, 200 mg. of DL-serine and 50 mg. of glycine per liter[9]; y, same as x with, however, 1200 mg. of DL-alanine[10] and with no liver extract; z, Medium II of Sauberlich and Baumann[11] with, however, half as much of each purine and of uracil. Media v and x were not used in this study; they are included for possible future reference.

Assays.—Standard curves covered the range of 0–50 γ of L-amino acid. The final *p*H of media prior to inoculation was 6.8–7.0 in each case except when arabinose (pectin sugar) was employed (media t–v). In these cases *p*H was 6.5; the lower *p*H minimized darkening due to autoclaving. Incubation was for 72 hr. at 37°, except for glycine assays in which incubation was completed at 48 hr. The standard alkali was 0.05 *N* sodium hydroxide solution, the end-point being observed with brom thymol blue.

For the figures of Table IV, assay was carried out at lower levels, in order to consume less material and to permit more replication. Standard curves covered the range of 0.0–2.0 γ of L-amino acid. By use of a buret graduated in 0.01-ml. divisions, a microtip, and 48-hr. incubation periods, it was possible to standardize the growth response to permit replication of samples at two levels.

In the tables all percentage figures represent g. of amino acid per 100 g. of material analyzed; AT = aminoid treatment, the subscript indicating the number of the treatment; AT_0 indicates the compositional value from the untreated hydrolyzate.

Results and Discussion

The recoverability of amino acids after one PTC treatment and aqueous hydrolysis is illustrated in Table I. Worthy of particular attention are the results with amino acids possessing chemically functional side-chains, such as glutamic acid and lysine. In no case is there found a decrement in excess of the variation typically observed for individual microbiological assays. Although PTC undoubtedly reacts with such side-chains as those of lysine, hydrolysis of the side-chain phenyl-

(1) Journal Paper No. J-2180 of the Iowa Agricultural Experiment Station, Project 863, supported in part by a grant from the National Institutes of Health, Public Health Service. Presented in part at the Iowa Academy of Science, April 18, 1952.

(2) S. W. Fox, T. L. Hurst and K. F. Itschner, THIS JOURNAL, **73**, 3573 (1951).

(3) K. A. Kuiken, C. M. Lyman and F. Hale, *J. Biol. Chem.*, **171**, 551 (1949).

(4) K. A. Kuiken, W. H. Norman, C. M. Lyman, F. Hale and L. Blotter, *ibid.*, **151**, 615 (1943).

(5) C. M. Lyman, O. Moseley, S. Wood, B. Butler and F. Hale, *ibid.*, **167**, 177 (1947).

(6) M. S. Dunn, L. E. McClure and R. B. Merrifield, *ibid.*, **179**, 11 (1949).

(7) C. M. Lyman, K. A. Kuiken, L. Blotter and F. Hale, *ibid.*, **157**, 395 (1945).

(8) B. F. Steele, H. E. Sauberlich, M. S. Reynolds and C. A. Baumann, *ibid.*, **177**, 533 (1949).

(9) M. N. Camien and M. S. Dunn, *ibid.*, **185**, 553 (1950).

(10) M. S. Dunn, S. Shankman, M. N. Camien, W. Frankl and L. B. Rockland, *ibid.*, **156**, 703 (1944).

(11) H. E. Sauberlich and C. A. Baumann, *ibid.*, **166**, 417 (1946).

thioureide in the absence of molecular α-carboxyl proceeds in such a manner as to obviate difficulties from this source.

TABLE I

RECOVERABILITY OF AMINO ACIDS FROM ACTH-79R AFTER ONE PTC TREATMENT AND AQUEOUS HYDROLYSIS

Amino acid	AT_0, %	AT_1, %
Arginine(ar)	16	16
Cystine(aq)	3.5	3.5
Glutamic acid(aq)	4.5	4.5
Histidine(mr)	3.3	3.8
Isoleucine(aq)	5.4	5.7
Leucine(aq)	10	10
Lysine(ms)	6.9	6.7
Methionine(fq)	2.1	2.1
Phenylalanine(bu)	3.9	3.7
Proline(bu)	3.7	3.3
Serine(fy)	15	13
Tyrosine(aq)	5.1	4.3
Valine(aq)	4.9	5.0

In Table II are presented results of sequential analyses of two preparations of ACTH fragments, each from the same original pool of material by the peptic method of White, Fierce and Lesh.[12] A and B each were reported as assaying about 50 × standard. Preparation B yielded a single spot upon chromatography in 17 different solvent systems.[13] Because of the paucity of sample, analytical values were obtained singly. Concordance of these values upon successive treatments was relied upon for replication. This was considered to be a more rigorous test of reproducibility than replication of values from the same treatment. In those instances (isoleucine, proline) in which decrements were recorded, the sequential assays were repeated, with the exception of phenylalanine in A. Close concordance between the values for preparations A and B also suggested that the method was accurate within the usual limits of microbial assay. In a few instances, it was possible to assay with two different microbes, a situation which provided a further check. The analytical figures have been entirely converted to numbers of residues, on the basis of isoleucine = 1.0, molecular weight of 4400. All of the figures in any one row were obtained from a simultaneous assay with a single standard curve. Concordance between different lines for any one amino acid served, therefore, as a further test of reproducibility. The results presented in Table II represent all those that were obtained and that met the requirements enumerated. These amino acids proved to be the ones that exhibited agreement with integral values in most cases.

Table II illustrates, as does Table I, that the PTC does not combine irreversibly with reactive side-chains. It may also be noted that except for near-integral decrements (indicated by underlines) values tended to remain constant through the successive selective fissions with dioxane–HCl. This is of particular interest in the case of serine, inasmuch as under some conditions aminoid serine is known to be labile.[14] As Table II shows, no significant decreases are recorded for aminoid serine following successive blocking with mildly alkaline phenyl isothiocyanate and selective fission with dioxane–HCl.[15]

It is noteworthy that isoleucine occurs in the

TABLE II

SEQUENTIAL RESIDUE ANALYSIS OF PEPSIN-DERIVED ACTH[12]

Amino acid	AT_0[a]	AT_1	AT_2	AT_3	AT_4
		Preparation A			
Histidine(mq)	1.1	1.1	1.0	1.0	1.0
			1.0	1.0	1.0
Isoleucine(aq)	1.0	1.0			
			1.0	1.0	0.1
Leucine(aq)	3.7	3.7			
			3.7	3.7	3.7
Lysine	2.8(fs)	2.8			
	2.8(ms)	2.8			
			1.9(ms)	1.9	1.9
			1.9(ms)	1.9	1.9
Phenylalanine(bt)	1.6	0.9			
			0.9	0.9	0.9
Serine(my)	1.4	1.5	1.5	1.5	1.5
Tyrosine(bt)	2.7	2.8			
			2.7	2.8	2.7
Valine(aq)	1.5	1.5	1.5		
				1.5	1.5
		Preparation B			
Arginine(fq)	2.4	2.4			
			1.8	1.8	1.9
	2.2				
Histidine(mq)	1.0				
		1.0			
			1.0	1.0	1.0
Isoleucine	1.0(aq)	1.0			
		1.0(aq)	1.0	1.0	0.1
	1.0(aq)			1.0	0.2
	1.0(bt)				
Lysine	2.1(fs)	2.0			
			2.0(fs)	2.0	2.0
		2.0(fs)			
	2.0(bt)		2.0(ms)	2.0	2.0
Phenylalanine(bt)	0.7				
	1.0				
	1.0				
		0.9	0.9	0.9	0.8
			0.9	0.9	0.9
Proline(bt)	2.0				
	2.0				
	2.0				
		2.2	2.2	2.0	1.1
			2.0	2.0	0.8
Serine(my)	3.1	3.2	3.2	3.2	3.2
Threonine(fz)	0.0	0.0	0.0	0.0	0.0
Tyrosine(bt)	2.7				
		2.8			
			2.8	2.8	2.8

[a] AT = aminoid treatment, the subscript indicating the number of the treatment. AT_0 indicates the compositional value from assay of the untreated hydrolyzate.

(12) W. F. White, W. L. Fierce and J. B. Lesh, *Proc. Soc. Exptl. Biol. Med.*, **78**, 616 (1951).

(13) W. F. White, personal communication.

(14) D. F. Elliott, *Biochem. J.*, **50**, 542 (1952).

(15) Qualitatively similar results with dioxane–HCl have been recorded: W. A. Landmann, M. P. Drake and J. Dillaha, THIS JOURNAL, **75**, 3638 (1953).

fourth position in each preparation. The residual relative approximate 10% of isoleucine is probably explainable on the basis that the corresponding PTH (phenyl thiohydantoin) is one of the few that the assay organism can partially utilize.[16]

The observation of the occurrence of isoleucine and proline both in the same position can be explained as gross heterogeneity (unlikely in view of the number of integral residual values), salt formation between two or more diverse peptides, or by a branched molecule. It is further of interest that the decrement in isoleucine corresponds closely to all of a whole residue, whereas that of proline appears to represent one of two proline residues. The material was insufficient to permit checking on whether or not two amino acids exist in each of the three prior positions.

To the extent that this kind of analysis represents a means of identification, it may be seen that preparations A and B are quite similar. Principal discrepancies are found for serine, lysine and phenylalanine.

The figures suggest that a phenylalanyl peptide is present in A and lacking in B. This primary difference seems explainable on the basis that pepsin is known to hydrolyze aminoid phenylalanine[17] and the postulate that the corresponding component is an impurity resulting from peptic action. The data for the phenylalanyl component of A provide the one instance in Table II of a decrement for which a repetition of the assay was not possible. A similar AT_0–AT_1 decrement was, however, observed for another pepsin-derived preparation, $AT_0 = 4.8\%$, $AT_1 = 2.0\%$. The data indicate a near-integral impurity in A, probably phenylalanyllysine or a small peptide containing this dipeptide residue. The presumed impurity should be small, inasmuch as most of the other figures are not significantly altered by the presence of this component. Contamination of a peptide of mol. wt. 4400 by an equimolar amount of phenylalanyllysine would, for example, alter other proportions by less than 7 relative per cent.

Both the stoichiometrically derived molecular weight and the high ratios of some amino acids to others indicate for preparation B molecular weights considerably in excess of the earlier suggestion of an average octapeptide.[18] One interpretation of all of these results is that the average octapeptide of Li[18] represented a mixture composed partly of larger molecules possessing major activity, but primarily of smaller molecules.

In Table III are given the amino acid contents of ACTH protein calculated to the nearest 0.1 residue, from the data of Mendenhall.[19]

A number of amino acid proportions for an acid-derived preparation of activity (100–150 × standard) greater[20] than that of pepsin-derived samples is included in Table IV. Some sequential values are also included. The ratios observed agree with Armour results (by papergrams) in the cases of histidine, leucine, lysine, serine, tyrosine, and for two amino acids present in weaker preparations but absent in this one, isoleucine and threonine. Disagreements of one residue are found for arginine, glutamic acid and glycine. Methionine, also, which has been found in the present sample, was not reported from papergrams run in the Armour Laboratories,[20] but has since come to light in studies of fragments of Corticotropin A.[13]

It is of interest to compare these ratios with those found in a pepsin-digested hormone preparation of reported activity 250–300 × standard[21] as well as the others mentioned. The high proportions of glutamic acid and glycine are found in both cases. The basic amino acids are, however, present in lower proportions in this material. Methionine is also accounted for in the Merck compositional assay. Isoleucine and threonine are lacking in all preparations of Corticotropin A or B. These amino acids seem, therefore, to be superfluous to the maximal activity of the hormone. Although the protein hormone[19] contains isoleucine and

TABLE III

AMINO ACID CONTENTS OF ACTH PROTEIN[19] IN RESIDUES

Amino acid	Microbial assay 1	Microbial assay 2	Column assay
Alanine	..	..	18.1
Arginine	11.3	11.8	13.5
Aspartic acid	11.4	10.8	11.8
Cystine	6.8	8.2	7.8
Glutamic acid	24.0	25.1	23.3
Glycine	24.1	..	26.4
Histidine	1.9	1.9	2.1
Isoleucine	5.3	5.9	5.2
Leucine	13.4	12.6	13.0
Lysine	7.7	7.2	8.3
Methionine	2.9	1.8	1.4
Phenylalanine	5.5	5.8	5.7
Proline	16.1	15.3	19.1
Serine	12.9	..	14.6
Threonine	6.1	4.0	7.6
Tyrosine	3.0	3.0	3.8
Valine	6.6	6.5	6.8

TABLE IV

SOME MOLAR RATIOS OF AMINO ACIDS IN A CORTICOTROPIN-A[20] SAMPLE

Amino acid	AT_0	AT_1	AT_2
Arginine(fr)	1.2	1.2	1.2
Glutamic acid(aw)	5.2	...	...
Glycine(bt)	4.1	3.5	...
Histidine(mr)	1.1	...	1.2
Isoleucine(aq)	0.1		
Leucine(aq)	1.9	...	...
Lysine(ms)	3.1	...	...
Methionine(fp)	1.0	...	...
Phenylalanine(bt)	2.0	2.0	...
Serine(my)	1.2	0.0	...
Threonine(fz)	0.0		
Tyrosine(bt)	0.9	1.0	0.4
	1.1	1.2	0.3

(16) W. F. Serat, M.S. Thesis, Iowa State College, 1953. The values obtained in aqueous solution for such PTH's are considerably larger than when they are recovered following the blocking treatment of a peptide and subsequent hydrolytic treatment.

(17) J. S. Fruton, M. Bergmann and W. P. Anslow, Jr., *J. Biol. Chem.*, **127**, 627 (1939).

(18) C. H. Li, *J. Endocrinol.*, **6**, xl (1950).

(19) R. M. Mendenhall, *Science*, **117**, 713 (1953).

(20) W. F. White, THIS JOURNAL, **75**, 503 (1953).

(21) N. G. Brink, G. E. Boxer, V. C. Jelinek, F. A. Kuehl, Jr. J. W. Richter and K. Folkers, *ibid.*, **75**, 1960 (1953).

threonine, the molar ratios otherwise exhibit an interesting similarity to the ratios reported in this paper.

In yet another acid-derived preparation of approximately 30 × standard activity,[22] no threonine nor isoleucine was found. As in Corticotropin A (Table IV) a terminal aminoid L-serine residue was also indicated; AT_0 = 3.2%, AT_1 = 0.0%. The possibility that the hormone is originally a cyclic molecule, in which the aminoid serine is particularly labile to some of the purification agents such as acetic acid (*cf.* ref. 14) cannot, however, be excluded. Indication of penultimate L-tyrosine has also been obtained (Table IV).[23] The L-seryl-L-tyrosyl sequence has also been tabulated for a less pure Corticotropin A in this study. The configurations of residues can be designated inasmuch as the microbes used respond only to the L-components of DL-serine and DL-tyrosine.

From the work reported in the literature and here, it is clear that "ACTH protein" of unit activity and average molecular weight of about 22,000 may be converted into material with average molecular weight of $^1/_{10}$ to $^1/_4$ that of the protein, yet with activity 100–250 times as great. For this fundamental problem there has been offered the explanation that (a) only 1% of the protein molecules are active and (b) there is a facile disaggregation of sibling molecules of which only a small fraction is potentially active.[21] Another explanation in accord with the facts suggests that a number of related peptides of diverse constitution and size are convertible by the organism into ACTH-active substances and that certain types of alteration of less active molecules uncovers much more active remnants. Such alteration might consist of disaggregation or a debridement of a precursor molecule. The consistent absence of isoleucine and threonine in the most active materials, plus the appearance of terminal serine agree with this postulate. The similarity in molar ratios of amino acids between Mendenhall's protein and Corticotropin A (Tables III and IV) also supports the idea that molecules with quantitatively disparate activities are much alike. Should this present hypothesis be correct, it might suggest for ACTH a hormonogen–hormone relationship which would be analogous to the zymogen–protease conversion.[24]

(22) E. B. Astwood, M. S. Raben, R. W. Payne and A. B. Grady, THIS JOURNAL, **73**, 2969 (1951).

(23) The type of result in which as much as 0.3–0.4 of a whole residue of tyrosine is recovered in a series (Table IV) has not been usual in application of the method in this Laboratory, and is receiving further study.

(24) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948.

The work described here permits further evaluation of a quantitative sequential method of peptide analysis on natural peptides. Other applications of the PTC[25] and the mother phenyl isocyanate technique[26] to insulin have been recorded.[27–30] Certain advantages and disadvantages are discernible for the particular subtractive, quantitative, sequential (dioxane–HCl) technique employed here.

The present method can be no more reliable quantitatively than the type of amino acid assay employed; indeed its principal limitations appear to be of this nature. When practiced without a guiding extractive study, fullest significance of results requires complete amino acid assays and will seldom, if ever, identify non-amino acid moieties. This last limitation is simultaneously a virtue in that such a method reveals its own defects more fully than a less comprehensive approach, a point deserving emphasis at this stage of knowledge in peptide analysis. Useful information can of course be obtained without complete analyses, and this technique has been used as a guide for extractive procedures as well as the reverse.[31] The difficulties with and need for a truly quantitative method have been expressed[2] and emphasized.[32] The data in this paper indicate fission by dioxane–HCl of only the terminal blocked residues during successive treatments, and no special difficulties due to reactive side-chains. In addition, lack of change as in proteolysis[33] or in studies of biological metamorphosis can be based upon positive data rather than upon the absence of negative data. The present method, also, is practiced without the losses that often accompany fractionation.[31]

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