[CONTRIBUTION FROM THE RESEARCH LABORATORIES, MERCK & CO., INC.]

Pituitary Hormones. VII. The Nature of Corticotropin-B

BY NORMAN G. BRINK, GEORGE F. BOXER, V. C. JELINEK, FREDERICK A. KUEHL, JR., JOHN W. RICHTER AND KARL FOLKERS

RECEIVED SEPTEMBER 8, 1952

Corticotropin-B appears to be a peptide of molecular weight in the range of 5000 to 7000. It contains fourteen common amino acids in amounts corresponding to a chain of some sixty amino acid units. The preponderance of basic amino acids reflects the known basic nature of corticotropin-B. The purity of corticotropin-B and the question of the presence or absence of a prosthetic group are discussed. Corticotropin-B is highly active clinically in rheumatoid arthritis. It possesses adrenal weight-increasing activity and causes melanophore expansion in frog skins.

Corticotropin-B, a substance of high adrenocorticotropic activity, was isolated from pepsin-digested concentrates of hog pituitary gland extracts by the methods described in preceding papers of this series.[1] The final steps in the isolation procedure involved the use of ion-exchange resins and then countercurrent distribution and yielded material of 250–300 u./mg. activity. As judged by its behavior in countercurrent distributions, the corticotropin-B so obtained behaved as an essentially homogeneous substance. Not more than 5% of foreign material was detected by this method. There were indications, however, of some additional loss of biological activity during the later stages of the distribution. Evidence obtained during studies of corticotropin-B in the ultracentrifuge, as described below, add further weight to the belief that corticotropin-B has been obtained in essentially pure form.

The acetate salt of pure corticotropin-B is a white, amorphous solid which is soluble in water and methanol, and insoluble in acetone, benzene, ether and other similar solvents. There can be no doubt of its gross polypeptide nature. Its infrared spectrum discloses only absorption of groups associated with peptides. In aqueous solution, the ultraviolet absorption spectrum exhibits a single maximum at about 2770 Å., $E^{1\%}_{1\,cm.}$ 19.9. This absorption may be ascribed to the presence of amino acids having aromatic nuclei. The acetate salt of corticotropin-B contains 17.6% of nitrogen, and less than 0.5% of sulfur. Corticotropin-B contains no phosphorus and no carbohydrate. It gives a very weak ninhydrin test.

The amino acid composition of a sample of corticotropin-B is summarized in Table I; this sample was from a countercurrent distribution. The values recorded, with the exception of that for tryptophan, were obtained by fractionation of an acid hydrolyzate on ion-exchange columns according to the method of Stein and Moore.[2] The tryptophan was determined separately by the method of Shaw and McFarlane.[3] Fourteen common amino acids were present; the corticotropin-B hydrolyzate contained no leucine, isoleucine, threonine or cystine. It is of interest that 99.5% of the total nitrogen in the acid hydrolyzate is accounted for by the thirteen amino acids and ammonia; the contribution of the tryptophan nitrogen in the acid hydrolyzate is of course included in the value for ammonia. On a weight basis, 86.5% of the preparation, not corrected for moisture or anion (acetate) content, was recovered as weights of the amino acids. The known basic character of corticotropin-B is clearly reflected in the preponderance of the basic amino acids, as indicated in Table I.

TABLE I

AMINO ACID COMPOSITION OF CORTICOTROPIN-B ISOLATED BY COUNTERCURRENT DISTRIBUTION

Amino acid	% of Weight of prepn.[a]	% Total N in the acid hydrolyzate	Best calcd. molar ratio
Glycine	4.4	6.4	6.0
Alanine	1.9	2.3	2.2
Valine	6.7	6.3	5.9
Leucine	None	..	...
Isoleucine	None	..	...
Proline	5.8	5.5	5.1
Serine	4.5	4.7	4.3
Threonine	None	..	...
Phenylalanine	4.7	3.1	2.9
Tyrosine	6.9	4.2	3.9
Cystine	None	..	...
Methionine	2.5	1.9	1.7
Aspartic acid	5.3	4.4	4.1
Glutamic acid	8.1	6.0	5.7
Histidine	3.1	6.6	2.0
Lysine	11.4	17.0	8.0
Arginine	13.4	28.0	6.5
Ammonia	0.5	3.1	2.9
Tryptophan	7.3[b]		3.7

[a] Weight of preparation uncorrected for moisture. [b] Determined separately.

Table II presents the results of amino acid analyses of hydrolyzates of corticotropin-B purified to the 300-u./mg. potency level by oxycellulose (sample 1) and Amberlite IRC-50 (sample 2) columns. In general, the compositions of these fractions were similar to each other and to that of corticotropin-B which had been purified by countercurrent distribution. Small discrepancies may be seen in the values for serine, phenylalanine, and glutamic acid, and perhaps others. This is not unexpected since, as has been noted,[1d] countercurrent distribution revealed the presence of other components in corticotropin-B samples isolated even at this activity level by the column techniques.

Corticotropin-B appears to contain little or no cysteine. Analysis of an acid hydrolyzate which

(1) (a) N. G. Brink, F. A. Kuehl, Jr., J. W. Richter, A. W. Bazemore, M. A. P. Meisinger, D. E. Ayer and K. Folkers, THIS JOURNAL, **74**, 2120 (1952); (b) A. W. Bazemore, J. W. Richter, D. E. Ayer, J. Finnerty, N. G. Brink and K. Folkers, *ibid.*, **75**, 1945 (1953); (c) J. W. Richter, D. E. Ayer, A. W. Bazemore, N. G. Brink and K. Folkers, *ibid.*, **75**, 1952 (1953); (d) F. A. Kuehl, Jr., M. A. P. Meisinger, N. G. Brink and K. Folkers, *ibid.*, **75**, 1955 (1953).

(2) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(3) J. L. D. Shaw and W. D. McFarlane, *Can. J. Research*, **16B**, 361 (1938).

TABLE II

AMINO ACID COMPOSITIONS OF SAMPLES PURIFIED BY COLUMN TECHNIQUES

Amino acid	% of total N in the hydrolyzate Sample 1	Sample 2	Best calcd. molar ratio Sample 1	Sample 2
Glycine	6.9	6.9	6.5	5.9
Alanine	2.4	2.6	2.1	2.1
Valine	5.4	6.7	4.8	5.6
Leucine	..	...	...	...
Isoleucine	..	...	...	...
Proline	7.0	6.2	6.4	5.2
Serine	3.1	3.1	2.9	2.6
Threonine	..	...	...	...
Phenylalanine	2.2	2.8	2.0	2.1
Tyrosine	4.4	4.9	4.0	4.1
Cystine	..	...	...	...
Methionine	1.8	1.6	1.6	1.5
Aspartic acid	4.8	4.7	4.3	4.0
Glutamic acid	6.0	6.1	5.4	5.1
Histidine	5.9	6.6	1.8	1.8
Lysine	16.7	16.0	7.6	6.9
Arginine	29.2	28.1	6.6	6.0
Ammonia	3.3	3.9	3.0	3.3
Total	98.8	100.2		

had been treated with performic acid[4] showed only a minute amount of cysteic acid, indicating a corresponding absence of cysteine in the original preparation.

On the basis of the amino acid analyses of corticotropin-B, molar ratios were calculated for the amino acids found to be present (Table I). From these values, which are gratifyingly near to whole number ratios, it may be inferred that the corticotropin-B molecule contains about sixty amino acid units, or an integral multiple thereof, and has a minimum molecular weight of the order of 6,000–7,000.

A sample of highly purified corticotropin-B was examined for its behavior in the ultracentrifuge. The material appeared to be of high purity, and gave a sedimentation constant (35°) of 5.3×10^{-14} cm./sec./dyne and a diffusion constant of 9.0×10^{-7} cm.^{2}/sec. The partial specific volume was determined to be 0.70, a value significantly lower than that of 0.75 usually assumed for proteins. The calculated molecular weight, using these values, is 5,200. This appears to be in good agreement with the figure suggested as a minimum molecular weight by the analytical findings.

In materials of the nature of corticotropin and corticotropin-B, the establishment of absolute purity is a task of large proportions. Corticotropin-B appeared to be of high purity in its ultracentrifugal behavior. It has behaved as a single substance in countercurrent distribution. Harfenist and Craig in their recent work on insulin[5] have demonstrated that a compound of this size can be purified and characterized by countercurrent distribution; and in one case they were able to show the heterogeneity of an insulin preparation which had appeared homogeneous by the criteria of the solubility method, electrophoresis and the ultracentrifuge. The agreement between the molecular weights of corticotropin-B observed in the ultracentrifuge and calculated from the amino acid composition could be cited as additional evidence of purity. Despite all this, it is clear that if other components having the same or nearly the same amino acid compositions, molecular weight and solvent solubility (*i.e.*, distribution coefficient) were present, they might well remain undetected. Inactivation products of corticotropin-B, altered from the parent structive in some subtle way, could well fit these specifications; a suggestion that this may be the case has been noted.[1d] Further study and the application of other criteria of purity or new fractionation techniques may help answer this question.

The possible presence of a small active prosthetic group in corticotropin or corticotropin-B has long been of interest. No proof has been found to demonstrate that such a group exists. Should such a prosthetic group occur in corticotropin-B, it would have to be of low molecular weight and contain so little nitrogen and so little characteristic ultraviolet or infrared absorption that these properties would be obscured by those of the gross peptide. Otherwise it would not have escaped detection.

There is no clear explanation in chemical terms for the reversible inactivation and reactivation of corticotropin-B by mild oxidation and reduction, respectively. A sulfhydryl disulfide oxidation–reduction equilibrium was early considered. The failure to demonstrate the presence of sulfhydryl groups in the molecule makes this unlikely. One might speculate on the possible role of an elusive, reactive prosthetic group in this connection, but again without any direct evidence.

The most highly purified preparations of corticotropin-B have shown activities of about 250 to 300 u./mg. in the adrenal ascorbic acid depletion assay in hypophysectomized rats. A sample of corticotropin-B was tested clinically in rheumatoid arthritis by Dr. Charles Ragan.[6] It was found when administered by continuous intravenous injection in a dose of 0.1 mg., to be equivalent in antiarthritic effect to 20 mg. of Armour Standard La-1-A in injected in the same fashion.

Discrepancies have been noted in the assay of adrenocorticotropic preparations by adrenal ascorbic acid depletion and adrenal weight gain in hypophysectomized rats, suggesting that these two effects may be mediated by different hormones.[7,8,9] Samples of corticotropin-B which had been purified to activities of about 250 u./mg. (adrenal ascorbic acid depletion assay) by ion-exchange treatment and countercurrent distribution were highly active in increasing adrenal weight and causing thymus atrophy in hypophysectomized rats. The identity or close relationship between corticotropin and the melanophore-expanding hormone has been postulated by Sulman[10] and independently confirmed by

(4) J. M. Mueller, J. G. Pierce, Helen Davoll and V. du Vigneaud, *J. Biol. Chem.*, **191**, 309 (1951).

(5) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952).

(6) Columbia University College of Physicians and Surgeons, Presbyterian Hospital, New York City.

(7) W. O. Reinhardt and C. H. Li, *Proc. Soc. Exptl. Biol. Med.*, **77**, 229 (1951).

(8) A. W. Moyer, J. Van der Scheer, H. Ritter, W. C. Tesar, J. B. Logan, J. J. Oleson and H. R. Cox, *ibid.*, **79**, 1 (1952).

(9) H. B. F. Dixon, M. P. Stack-Dunne, F. G. Young and D. B. Cater, *Nature*, **168**, 1084 (1951).

(10) F. G. Sulman, *ibid.*, **169**, 588 (1952).

Johnsson and Högsberg.[11] However, Geschwind, Reinhardt and Li[12] do not accept this conclusion. It has been observed here that, although quantitative values are not available, highly purified corticotropin-B caused melanophore expansion in excised frog skin, even at low concentrations where crude corticotropin was ineffective. The details of these studies will be reported elsewhere.

The recent preparations here[1] and elsewhere[13,14] of unhydrolyzed corticotropin concentrates many times more active than the protein described in 1943 as the adrenocorticotropic hormone[15,16] have inevitably raised questions as to the nature of the reportedly pure protein preparation. The ease with which corticotropin can be removed from the "protein" can be interpreted by a hypothesis that the "protein" consists of an aggregate of proteinaceous units having a more or less characteristic composition, but still capable of easy separation into the component parts as, for example upon dialysis, ultra-filtration, acetic acid extraction or adsorption on oxycellulose or resin. A second interpretation is that the "protein" preparation considered homogeneous actually contained 1% or less of the active principle as a trace constituent, an amount impossible to detect in material of this nature by the physical criteria which were used.

Experimental

Acid Hydrolysis Prior to Analytical Separation of Amino Acids.—Samples weighing 8 to 10 mg. were dissolved in about 0.05 ml. of double-distilled water, followed by the addition of twice redistilled constant boiling hydrochloric acid. The mixtures were boiled under reflux for 18 hours in an atmosphere of nitrogen. Excess hydrochloric acid was removed by repeated evaporation *in vacuo* under nitrogen, and the residues finally dried *in vacuo*. The dried residues were taken up in the buffer of *p*H 3.42 described by Moore and Stein[2] containing thiodiglycol and Versene. One-half ml. aliquot portions containing the equivalent of between 2.0 and 2.5 mg. of unhydrolyzed material were used for chromatography. Total nitrogen in the hydrolyzates was determined by a modification of the Nessler reaction.[17]

Performic Acid Oxidation of Corticotropin-B.—A performic acid solution was prepared by adding 0.5 ml. of 30% hydrogen peroxide solution to 4.5 ml. of 88% formic acid and allowing the mixture to stand at room temperature for 25 minutes. A 10-mg. quantity of corticotropin-B was dissolved in 5 ml. of this solution and the reaction mixture was kept at −5° for 45 minutes. It was then diluted with 20 ml. of water and lyophilized. The solution in water and lyophilization were repeated. The product, which had a bioactivity of less than 25 u./mg. was hydrolyzed with acid as described above. Only a trace of cysteic acid was found in the hydrolyzate.

Acknowledgment.—The authors are indebted to Mr. R. N. Boos and his associates for the microanalyses, to Dr. N. R. Trenner and co-workers for the infrared spectra and to Mr. Arthur Rosenberg for the ultracentrifuge determinations. They wish to thank Dr. C. A. Winter of the Merck Institute for Therapeutic Research for his studies on the adrenal weight and melanophore-expanding properties of corticotropin-B. The assistance of Messrs. D. E. Ayer and M. A. P. Meisinger in the preparation of samples of corticotropin-B for this work is gratefully acknowledged.

(11) S. Johnsson and B. Högsberg, *Nature*, **169**, 286 (1952).

(12) I. I. Geschwind, W. D. Reinhardt and C. H. Li, *ibid.*, **169**, 1061 (1952).

(13) H. B. F. Dixon, S. Moore, M. P. Stack-Dunne and F. G. Young, *ibid.*, **168**, 1044 (1951).

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

(15) C. H. Li, H. M. Evans and M. E. Simpson, *J. Biol. Chem.*, **149**, 413 (1943).

(16) G. Sayers, A. White and C. N. H. Long, *ibid.*, **149**, 425 (1943).

(17) W. W. Umbreit, K. H. Burris and S. F. Stauffer, "Manometric Techniques and Related Methods for the Study of Tissue Metabolism," Burgess Publishing Co., Minneapolis, Minn., 1945, p. 103.

RAHWAY, NEW JERSEY

[CONTRIBUTION FROM THE RADIATION LABORATORY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA]

Phosphorus and Photosynthesis. I. Differences in the Light and Dark Incorporation of Radiophosphate[1]

BY M. GOODMAN, D. F. BRADLEY AND M. CALVIN

RECEIVED SEPTEMBER 22, 1952

The distribution of radioactivity in metabolites of photosynthetic algae exposed to $KH_2P^{32}O_4$ in light and dark has been determined by paper chromatography and radioautography. The large relative increase of adenosine triphosphate in the dark and of 3-phosphoglyceric acid in the light is discussed in relation to the mechanism of phosphorylation of photosynthetic intermediates. Evidence is given indicating that adenosine triphosphate is the first isolable product formed from inorganic phosphate, and that uridine diphosphate glucose and adenosine diphosphate are active in phosphate metabolism.

Since the majority of significant photosynthetic intermediates are phosphorylated,[2-5] one might expect that there would be differences in the steady-state distribution of phosphate among intermediate metabolites in green plants depending on whether they are photosynthesizing or merely respiring. Furthermore, there might be differences in the relative rates at which entering phosphate is distributed among these same intermediates. However, fractionation methods[6-11] that have heretofore been employed in such studies have not per-

(1) The work described in this paper was sponsored by the U. S Atomic Energy Commission.

(2) M. Calvin and A. A. Benson, *Science*, **107**, 476 (1948).

(3) A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, THIS JOURNAL, **72**, 1710 (1950).

(4) J. G. Buchanan, *et al.*, "Phosphorus Metabolism," Vol. II, Johns Hopkins Press, Baltimore, Md., 1952.

(5) E. W. Fager, J. L. Rosenberg and H. Gaffron, *Federation Proc.*, **9**, 535 (1950).

(6) M. D. Kamen and S. Spiegelman, *Cold Spring Harbor Symposia on Quantitative Biology*, **13**, 151 (1948).

(7) R. L. Emerson, J. F. Stauffer and W. W. Umbreit, *Amer. J. Bot.*, **31**, 107 (1944).

(8) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minn., 1945, Chap. XV.

(9) S. Aronoff and M. Calvin, *Plant Physiology*, **23**, 351 (1948).

(10) H. Gest and M. D. Kamen, *J. Biol. Chem.*, **176**, 299 (1948).

(11) W. Simonis and K. H. Grube, *Z. Nat.*, **7b**, 194 (1952).