Adrenocorticotropins. 44. Total Synthesis of the Human Hormone by the Solid-Phase Method

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Abstract: The total synthesis of human ACTH (39 amino acid residues) has been accomplished by the solid-phase method. The identity of the synthetic product with the natural hormone was established by amino acid analysis, chromatography on carboxymethylcellulose, partition chromatography on Sephadex G-50, optical rotation, circular dichroism spectra, electrophoresis on both paper and polyacrylamide, electrophoretic patterns of both chymotryptic and tryptic digests, as well as by three biological tests. Two new amino acid derivatives, N^{α}-Boc-N^{ϵ}-(*o*-bromobenzyloxycarbonyl)lysine and N^{α}-Boc-*O*-(2,6-dichlorobenzyl)tyrosine, were employed for the synthesis.

As part of efforts directed toward the synthesis of adrenocorticotropin (ACTH)[1] and its analogs the nonadecapeptide α^{1-19}-ACTH has been synthesized[2] by the solid-phase method.[3] In order to extend the scope of this effort and apply recent improvements in solid-phase techniques, the total synthesis of the α-ACTH molecule was undertaken. The human hormone (α_h-ACTH) was selected. The proposed structure of this hormone[4] has recently been revised[5,6] (see Figure 1) and syntheses by conventional methods of the original structure[7] and the revised structure[8,9] have been reported. We now describe the solid-phase synthesis of α_h-ACTH in highly purified form with the full biological activity of the natural hormone.

Before undertaking the synthesis it was necessary to reexamine standard procedures of solid-phase synthesis. We have recently observed in some syntheses that severe irreversible chain termination occurs as chain length increases. The difficulty was largely resolved by a modification of the Loffet procedure[10] of attachment of the first Boc-amino acid as its tetramethylammonium salt to chloromethylated polymer. Thus, the tetramethylammonium salt of Boc-phenylalanine was allowed to react with chloromethylated polymer (0.72 mmol of Cl/g) in dimethylformamide at room temperature in a manner similar to that described by Marglin[11] for the standard procedure of attachment. Boc-phenylalanyl polymer was obtained with good substitution[12] (*ca.* 0.43 mmol of Phe/g). The optical purity of the phenylalanine attached in this manner was checked by synthesis of the dipeptide Glu-Phe with the Boc-phenylalanyl polymer, cleavage[13] of the dipeptide from the resin in HF, and analysis of the resulting product by the sensitive procedure of Manning and Moore.[14] Only one major peak corresponding to L-Glu-L-Phe was found, and the maximum amount of L-Glu-D-Phe that could have been present was about 0.2%. In addition, the dipeptide was completely digested by LAP.

For synthesis, the attachment of glutamic acid to phenylalanyl polymer was purposely limited to 0.28 mmol/g in order to reduce the load on the resin and to select the most sterically favorable sites. Uncoupled amino groups were then covered by acetylation with acetic anhydride.

Since N^{α}-Boc protection was used throughout the synthesis, a suitable selection of side-chain protecting groups was required. For histidine and arginine, N^{im}-Boc and N^{G}-tosyl protections, respectively, were chosen based on our previous experience.[15] Since Z protection for lysine is known to be unstable,[16] the Z(*o*-Br) protecting group[17] was used since it is about 60-fold more stable than Z. It has also been shown that Bzl protection of tyrosine is unstable.[17-19] The Bzl(2,6-Cl_2) protecting group[17] was employed since it is about 50-fold more stable than Bzl. For aspartic acid, serine, and glutamic acid, Bzl protection was used.

The Boc-Glu(Bzl)-Phe resin (3.2 g) was placed in a Beckman Model 990 peptide synthesizer and the

(1) Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, 247, 977 (1971). Other abbreviations used are: ACTH, adrenocorticotropic hormone; CMC, carboxymethylcellulose; DCC, *N,N'*-dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; DMF, dimethylformamide; DIA, diisopropylethylamine; tlc, thin layer chromatography; LAP, leucine aminopeptidase; cAMP, cyclic adenosine 3',5'-monophosphate; CD, circular dichroism.

(2) J. Blake, K.-T. Wang, and C. H. Li, *Biochemistry*, **11**, 438 (1972).

(3) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).

(4) T. H. Lee, A. B. Lerner, and V. Buettner-Janusch, *J. Biol. Chem.*, **236**, 2970 (1961).

(5) L. Graf, S. Bajusz, A. Patthy, E. Barat, and G. Csek, *Acta Biochim. Biophys.*, **6**, 415 (1971).

(6) B. Riniker, P. Sieber, and W. Rittel, *Nature (London), New. Biol.*, **235**, 114 (1972).

(7) S. Bajusz, K. Medzihradsky, Z. Paulay, and Sz. Lang, *Acta Chim. Acad. Sci. Hung.*, **52**, 335 (1967).

(8) L. Kisfaludy, M. Low, T. Szirtes, I. Schon, M. Sarkozi, S. Bajusz, A. Turan, R. Beke, A. Juhasz, L. Graf, and K. Medzihradszky, presented at the 3rd American Peptide Symposium, Boston, Mass., June 1972.

(9) P. Sieber, W. Rittel, and B. Riniker, *Helv. Chim. Acta*, **55**, 1243 (1972).

(10) A. Loffet, *Int. J. Protein Res.*, **3**, 297 (1971).

(11) A. Marglin, *Tetrahedron Lett.*, 3145 (1971).

(12) It is signifidant to note that Boc-Phe polymer prepared by standard triethylamine procedures retains picric acid in the Gisin amine test (B. F. Gisin, *Anal. Chim. Acta*, **58**, 248 (1972)). Boc-Phe polymer prepared by the modified Loffet procedure retains no picric acid after completion of this test and supports the conclusion of Loffet[10] that formation of quarternary ammonium groups is thereby avoided.

(13) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jap.*, **40**, 2164 (1967); J. Lenard and A. B. Robinson, *J. Amer. Chem. Soc.*, **89**, 181 (1967).

(14) J. M. Manning and S. Moore, *J. Biol. Chem.*, **243**, 5591 (1968).

(15) D. Yamashiro, J. Blake, and C. H. Li, *J. Amer. Chem. Soc.*, **94**, 2855 (1972).

(16) A. Yaron and S. F. Schlossman, *Biochemistry*, 7, 2673 (1968).

(17) D. Yamashiro, R. L. Noble, and C. H. Li, presented at the 3rd American Peptide Symposium, Boston, Mass., 1972.

(18) D. Yamashiro and C. H. Li, *Int. J. Peptide Protein Res.*, **4**, 181 (1972).

(19) B. W. Erickson and R. B. Merrifield, presented at the 3rd American Peptide Symposium, Boston, Mass., 1972.

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-
5 10
Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-
15 20
Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-
25 30
Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH
35 39

Figure 1. Structure of α_h-ACTH; see ref 3–5.

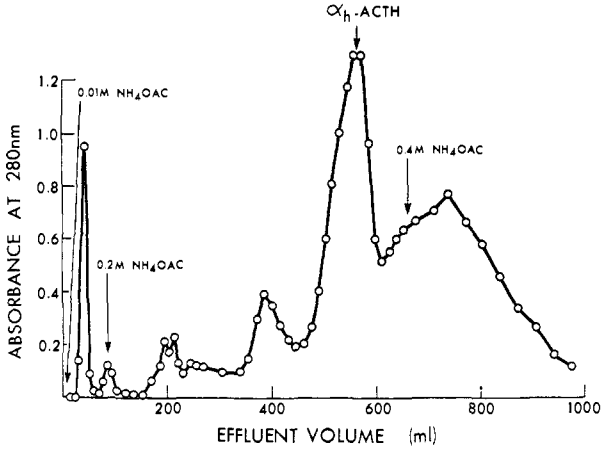

Figure 2. Carboxymethylcellulose chromatography of crude synthetic α_h-ACTH: sample, 202 mg treated with dithiothreitol; yields, 350–430 ml, 12 mg; 490–600 ml, 68 mg; 601–940, 77 mg.

remaining 37 amino acid residues were attached according to the schedules shown in the Experimental Section. The exception to the schedules occurred for the removal of the Boc group located on the amino group of tryptophan. This was accomplished by treatment with 25% TFA in CH_2Cl_2 for 30 min followed by a 6-min treatment with 50% reagent. No additives to the deprotecting agent were employed at any stage even after incorporation of the tryptophan residue.

A portion (1.00 g) of the finished peptide resin was treated in HF[13] in the presence of anisole for removal of all protecting groups and the solid support. The resulting peptide material was passed through Dowex 1-X4 (acetate form), lyophilized, and then chromatographed on Sephadex G-10 in 1 *N* acetic acid in which only one peak was detected. The isolated peptide material (346 mg) was subjected to gel fitration on Sephadex G-25 in 0.5 *N* acetic acid in which a small unretarded peak was followed by a major peak corresponding to the desired product. The major peak material (202 mg) was treated with dithiothreitol in 0.1 *N* acetic acid for 21 hr at 50° to convert any methionine sulfoxide to methionine[20] and then chromatographed on carboxymethylcellulose[21] (Figure 2) as previously described.[22] The position of the large major peak (68 mg) was very close to that[22] of natural ACTH. Rechromatography of this material (20 mg) on CMC gave the results shown in Figure 3, a single

(20) J. Ramachandran, unpublished observations.
(21) E. A. Peterson and H. A. Sober, *J. Amer. Chem. Soc.*, **78**, 751 (1956).
(22) B. T. Pickering, R. N. Andersen, P. Lohmar, Y. Birk, and C. H. Li, *Biochim. Biophys. Acta*, 74, 763 (1963). The position and symmetry of a peak on carboxymethylcellulose chromatography of either synthetic or natural α_h-ACTH is dependent on sample size.

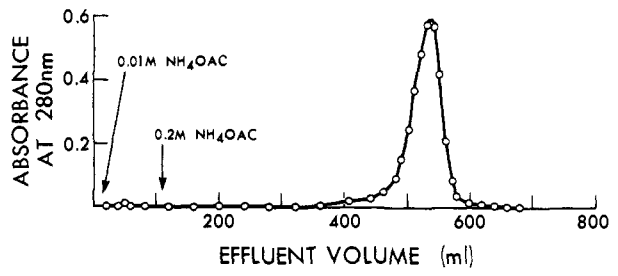

Figure 3. Rechromatography of partially purified synthetic α_h-ACTH on carboxymethylcellulose: sample, 20 mg; yield, 482–570 ml, 17 mg.

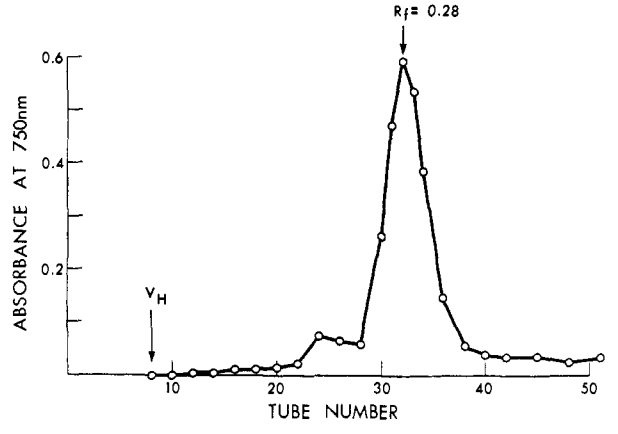

Figure 4. Partition chromatography on Sephadex G-50 of natural α_h-ACTH; absorbance measured by Folin–Lowry analysis; V_H = hold-up volume.

peak with an elution maximum of 537 ml. CMC chromatography of the natural hormone[23] (25 mg) under identical conditions gave an elution maximum of 540 ml. Since rechromatography of the synthetic material on CMC gave only a single peak it was evident that this procedure would not effect any further purification. We therefore devised a new procedure for partition chromatography on Sephadex G-50 based on previous techniques[24] worked out on Sephadex G-25. The solvent system 1-butanol–pyridine–0.1% aqueous acetic acid (5:3:11) previously employed in countercurrent distribution of adrenocorticotropins[25] was utilized. When natural α_h-ACTH (5 mg) was chromatographed under these conditions the results shown in Figure 4 were obtained, the hormone emerging as a sharp symmetrical peak with R_f 0.28. Chromatography of synthetic material (32 mg) from the CMC purification step gave results shown in Figure 5 with a major peak at R_f 0.26. Isolation of material represented by this peak gave 12 mg of highly purified synthetic preparation.

Rechromatography of a sample (3 mg) of the synthetic preparation by the partition chromatography gave a single symmetrical peak (Figure 6) with R_f 0.27, practically identical with that of natural hormone. Amino acid analysis of the highly purified synthetic preparation was in agreement with expected values (Table I). Paper electrophoresis at pH 3.7 gave a

(23) The natural hormone was isolated from fresh human pituitaries by a procedure previously described.[22]
(24) D. Yamashiro, *Nature (London)*, **201**, 76 (1964).
(25) J. S. Dixon, T.-B. Lo, and C. H. Li, *Arch. Biochem. Biophys.*, **92**, 296 (1961).

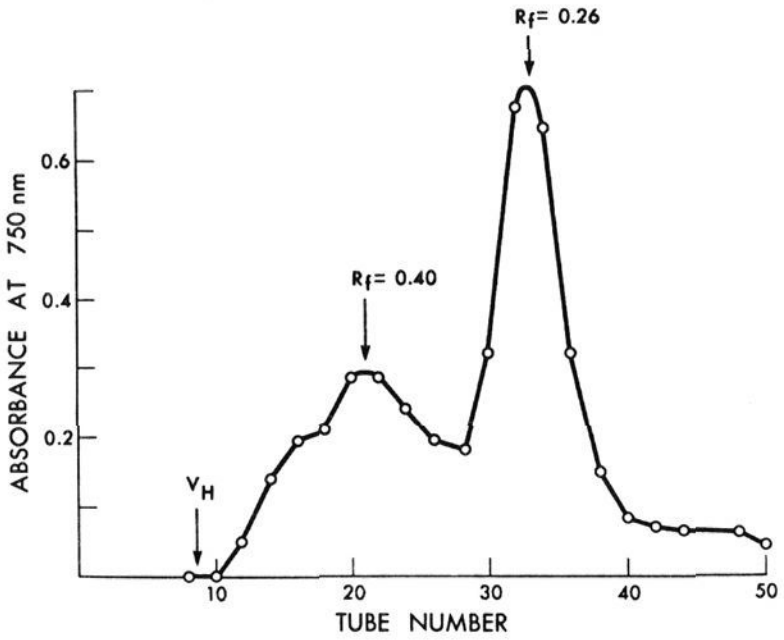

Figure 5. Partition chromatography on Sephadex G-50 of partially purified synthetic α_h-ACTH; absorbance measured by Folin–Lowry analysis; V_H = hold-up volume.

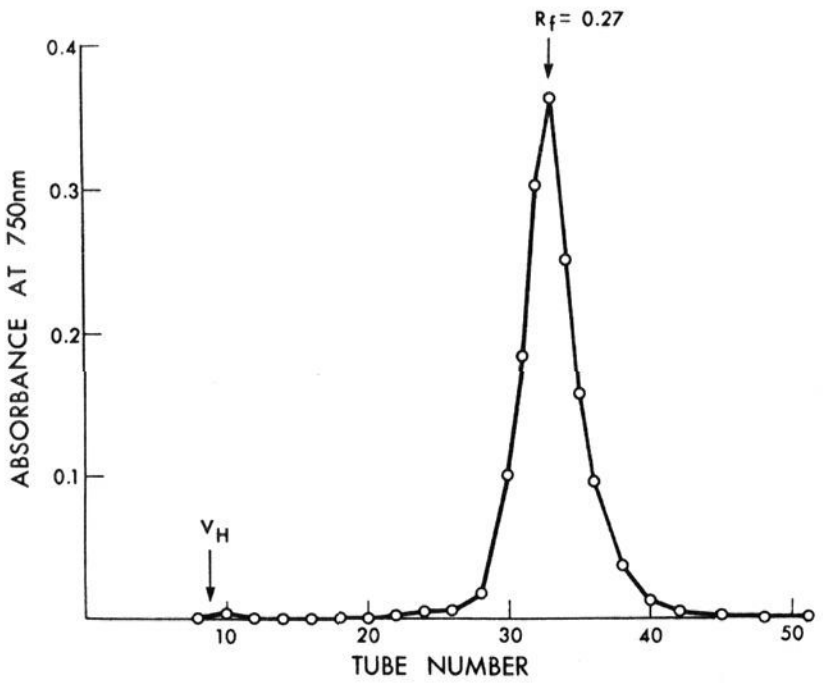

Figure 6. Partition chromatography on Sephadex G-50 of highly purified synthetic α_h-ACTH; absorbance measured by Folin–Lowry analysis; V_H = hold-up volume.

Table I. Amino Acid Analyses of Synthetic α_h-ACTH

Amino acid	Found	Calcd[a]
Lys	4.0	4
His	1.0	1
Arg	3.0	3
Asp	2.1	2
Ser	2.7	3
Glu	5.2	5
Pro	3.8	4
Gly	2.9	3
Ala	3.0	3
Val	3.0	3
Met	0.9	1
Leu	1.0	1
Tyr	2.0	2
Phe	3.1	3
Tryptophan[b]	1.1	1

[a] See Figure 1. [b] Determined spectrophotometrically; see G. Beavan and E. Holiday, *Advan. Protein Chem.*, **7**, 319 (1962).

single spot (Figure 7) paralleling that given by natural hormone. The behavior of the synthetic material in electrophoresis on polyacrylamide gel at pH 4.5 was identical with that of natural hormone (Figure 8).

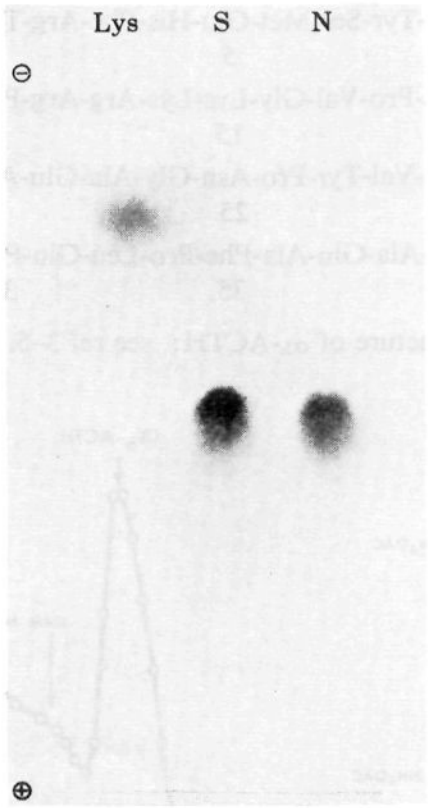

Figure 7. Paper electrophoresis at pH 3.7 of synthetic (S) and natural (N) α_h-ACTH.

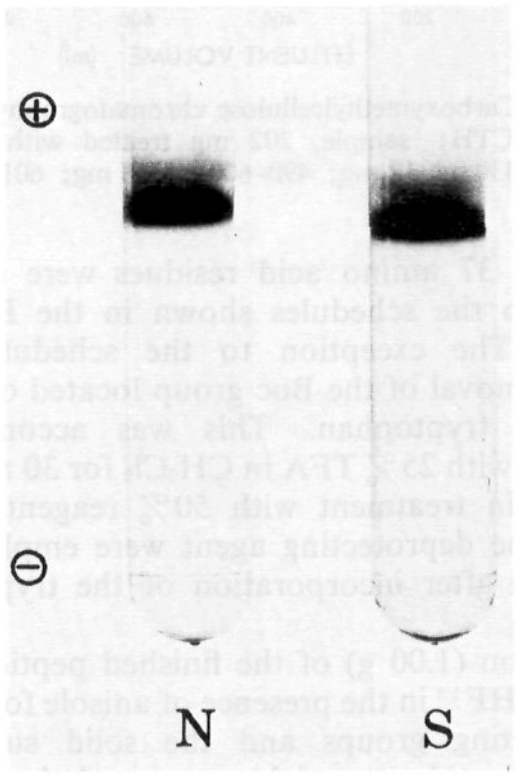

Figure 8. Electrophoresis of natural (N) and synthetic (S) α_h-ACTH on polyacrylamide gel at pH 4.5; samples, natural, 90 μg; synthetic, 80 μg. Detection by staining with Amido-Schwarz dye.

Electrophoretic patterns of a tryptic digest as well as a chymotryptic digest of synthetic material were also identical with those obtained with natural hormone (Figure 9). The circular dichroism spectra of the synthetic and natural hormones are virtually identical as shown in Figure 10. In addition, the synthetic material and the natural hormone were indistinguishable from each other by measurements of their optical rotatory dispersion (Table II) and ultraviolet absorption spectra.

The *in vivo* steroidogenic activity of the synthetic preparation does not differ significantly from that of the natural hormone when assayed by the procedure of Vernikos-Danellis, *et al.*,[26] as shown in Table III. The synthetic preparation is equally active in com-

(26) J. Vernikos-Danellis, E. Anderson, and L. Trigg, *Endocrinology*, **79**, 624 (1966).

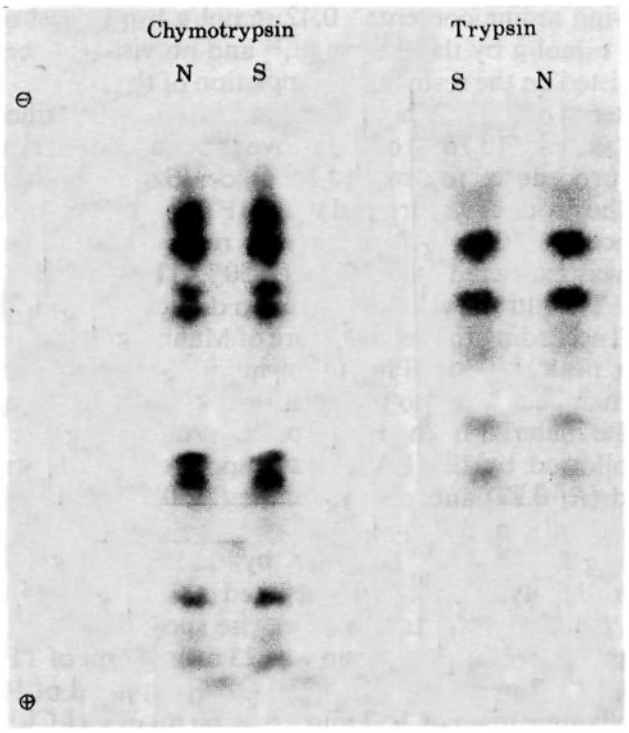

Figure 9. Paper electrophoresis at pH 6.9 of natural (N) and synthetic (S) α_h-ACTH after treatment with chymotrypsin (left) and with trypsin (right).

Table II. Optical Rotation of Natural and Synthetic α_h-ACTH

λ, nm	$[\alpha]^{27}\lambda$, deg	
	Natural	Synthetic
589	−97.8	−99.0
400	−252	−244
300	−641	−642
230	−1910	−2260
208	−7700[a]	−8240[a]

[a] These values represent those for the minimal rotation.

Table III. Biological Activities of Synthetic α_h-ACTH

Biological tests	α_h-ACTH	
	Synthetic	Natural
In vivo steroidogenic potency,[a] I.U./mg	140 (100–194) [3]	156 (121–196) [3]
Lipolytic activity[b] in		
isolated rat fat cells	10.4 ± 1.5 [4]	10.5 ± 3.5 [4]
isolated rabbit fat cells	40 [1]	40 [1]
Stimulation[c] of cAMP production in isolated rat adrenal cells	95 (85–106) [1]	100 [1]

[a] Numbers in parentheses represent 95% confidence limit; numbers in brackets represent the number of tests. [b] Concentrations, ng/ml, at one-half maximal response. [c] The potency of natural α_h-ACTH was assumed to be 100.

parison with the natural hormone for the ability to stimulate cAMP synthesis in isolated rat adrenal cells and lipolysis in isolated rat and rabbit fat cells (Table III) as estimated by procedures[27,28] previously described from this laboratory. Thus, these results demonstrate that the solid-phase method together with available techniques for purification can be used for the synthesis of a biologically active polypeptide of the size and complexity of ACTH.

Experimental Section

Melting points were determined on a Fisher-Johns block and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Thin-layer chromatography (tlc) was run on silica gel in the following solvents: chloroform–methanol, 1:1 (CM); 1-butanol–acetic acid–water, 4:1:1 (BAW). Trifluoroacetic acid, diisopropylethylamine, and DCC were distilled before use. Dioxane was distilled from sodium and stored at 4°. Dichloromethane was purified[29] by the procedure of Maryott, *et al.*,[30] and distilled from P_2O_5. A methanolic solution of tetramethylammonium hydroxide was obtained by passing a solution of tetramethylammonium bromide (2.46 g) in methanol (100 ml) through a 50-ml bed of Dowex 1-X4 (50–100 mesh, free base form in methanol). Styrene–divinylbenzene resin (Bio-Beads S-X1, 200–400 mesh, Bio-Rad Laboratories) was sieved on U. S. Standard screens to obtain the 200–270 mesh fraction. This sieved fraction was chloromethylated in $CHCl_3$ by standard procedures[3] to give a substitution of 0.72 mmol of Cl/g.

CMC chromatography[21] was performed in a 1.0 × 55 cm column with an initial buffer of 0.01 *M* NH_4OAc of pH 4.5. A gradient with respect to pH and salt concentration was effected by introducing the appropriate buffers (see Figures 2 and 3) through a 500-ml mixing chamber containing the starting buffer.

Partition Chromatography on Sephadex G-50. Sephadex G-50 Fine (Pharmacia Fine Chemicals, Inc.) was sieved on U. S. Standard screen No. 325, and the material passed through was used. Columns were packed in water under a flow rate of about 1 ml per min per cm^2. A column was equilibrated with the lower phase of the solvent system[31] 1-butanol–pyridine–0.1% aqueous acetic acid (5:3:11) followed by equilibration with the upper phase as described previously.[24] The peptide was dissolved in the upper phase (3 ml) and chromatographed by elution with the upper phase. Peptide material was detected by the Folin–Lowry procedure.[32] For isolation, appropriate fractions were mixed with an equal volume of water, evaporated *in vacuo* to low volume, and lyophilized. It was essential to use a freshly packed column for each experiment.

***p*-Nitrophenyl *o*-Bromobenzyl Carbonate.** 2-Bromobenzyl alcohol was prepared from 2-bromobenzaldehyde (Aldrich Chemical Co.) by reduction with sodium borohydride in absolute ethanol. The alcohol was used to prepare the title compound by a procedure analogous to that described for the preparation of the corresponding *p*-methoxybenzyl ester[33] with the exception that ethyl acetate was used in place of ether for extraction. From 10.8 g (58 mmol) of the alcohol and 9.7 g (48 mmol) of *p*-nitrophenyl chloroformate (Eastman) the product after recrystallization from hot absolute ethanol weighed 13.4 g (79% yield), mp 94–95°.

Anal. Calcd for $C_{14}H_{10}NO_5Br$ (352.16): C, 47.75; H, 2.86; N, 3.98. Found: C, 48.07; H, 2.89; N, 4.63.

N^{ϵ}-(*o*-Bromobenzyloxycarbonyl)lysine. To a boiling solution of lysine hydrochloride (22.25 g, 122 mmol) in 400 ml of water was

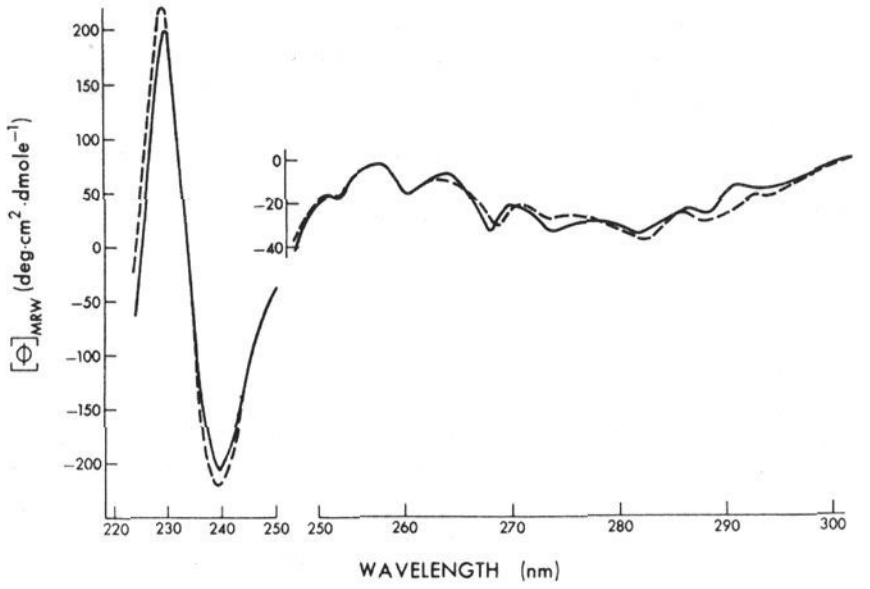

Figure 10. Circular dichroism spectra of natural (——) and synthetic (- - -) α_h-ACTH.

(27) W. R. Moyle, Y. C. Kong, and J. Ramachandran, *J. Biol. Chem.*, in press.
(28) J. Ramachandran and V. Lee, *Biochem. Biophys. Res. Commun.*, **38**, 307 (1970).
(29) K. Brunfeldt and T. Christensen, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **19**, 345 (1972).
(30) A. A. Maryott, M. E. Hobbs, and P. M. Gross, *J. Amer. Chem. Soc.*, **63**, 659 (1941).
(31) L. C. Craig and W. Konigsberg, *J. Org. Chem.*, **22**, 1345 (1957).
(32) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(33) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

added 16.4 g of basic copper carbonate. After 10 min at the boiling point the solution was cooled and insoluble material was filtered off. The filtrate was diluted with 1.2 l. of water and 4.9 l. of DMF. *p*-Nitrophenyl *o*-bromobenzyl carbonate (50 g, 142 mmol) and $NaHCO_3$ (22.3 g, 265 mmol) were added, and the mixture was stirred for 2 days. The blue solid was collected and washed with water and ethanol. The solid was dissolved in boiling water (6 l.) in the presence of (ethylenedinitrilo)tetraacetic acid disodium salt (*ca.* 50 g). The solution was cooled and then stored at 4° overnight. The colorless product was collected and washed with water and ethanol: yield, 42 g (96%); mp 220–223°; tlc (BAW) R_f 0.50; $[\alpha]^{24}D$ +9.6° (*c* 1.09, 80% acetic acid).

Anal. Calcd for $C_{14}H_{19}N_2O_4Br$ (359.24): C, 46.81; H, 5.33; N, 7.80. Found: C, 46.62; H, 5.10; N, 7.94.

Dicyclohexylamine Salt of N^{α}-Boc-N^{ϵ}-(*o*-bromobenzyloxycarbonyl)lysine. N^{ϵ}-(*o*-Bromobenzyloxycarbonyl)lysine (4.93 g, 13.7 mmol) was converted to the N^{α}-Boc derivative by standard procedures.[34] The oily product (5.0 g), tlc (CM) R_f 0.6, was dissolved in ether (50 ml) and mixed with 2.0 g (11 mmol) of dicyclohexylamine. The crystalline salt was obtained from ether–petroleum ether after storage at 0° for 1 week: yield, 5.38 g. Recrystallization from ether–petroleum ether overnight at room temperature gave 4.84 g (55%): mp 106–108°; $[\alpha]^{24}D$ +6.3° (*c* 2.44, $CHCl_3$).

Anal. Calcd for $C_{31}H_{50}N_3O_6Br$ (640.68): C, 58.21; H, 7.87; N, 6.56. Found: C, 57.88; H, 7.75; N, 6.29.

For establishment of optical purity a sample (238 mg) was treated with HF (10 ml) at 0° for 30 min in the presence of anisole (0.20 ml). After removal of HF at 0° the residue was dried *in vacuo* and taken up in 10 ml of 0.1 *N* HCl. The aqueous solution was washed with two 10-ml portions of ether and evaporated to dryness. The residue was extracted with 5 *N* HCl (4 ml); the insoluble salt was filtered off and washed with 5 *N* HCl (6 ml). The filtrate was evaporated to dryness. Quantitative amino acid analysis[35] for lysine gave a 99% yield. Determination of optical rotation gave $[\alpha]^{24}D$ +25.0° (*c* 1.09, 5 *N* HCl) based on the amount of lysine obtainable from the starting sample. Lysine hydrochloride treated in the same manner gives $[\alpha]^{24}D$ +24.6° (*c* 1, 5 *N* HCl).

***O*-(2,6-Dichlorobenzyl)tyrosine.** A solution of tyrosine (5.02 g, 27.7 mmol) in 28 ml of 2 *N* NaOH was mixed with a solution of $CuSO_4 \cdot 5H_2O$ (3.4 g) in 14 ml of water, heated to 60°, and then cooled to room temperature. After dilution with methanol (120 ml) 2,6-dichlorobenzyl bromide (9.0 g, 37.5 mmol) was added, and the mixture was stirred for 15 hr. The solid was collected and washed with 100 ml of 25% aqueous methanol, 100 ml of methanol, and 100 ml of acetone. The blue solid was dissolved in 1 l. of 50% ethanol at the boiling point in the presence of 10 g of (ethylenedinitrilo)tetraacetic acid disodium salt. The hot solution was decanted from any insoluble matter and stored at 4° overnight. The product was collected and washed with water and ethanol: yield, 4.0 g (43%); mp 192–196°. For analysis a sample was recrystallized from 50% ethanol: mp 200–203°; tlc (BAW) R_f 0.65; $[\alpha]^{24}D$ −10.6° (*c* 2, 80% acetic acid).

Anal. Calcd for $C_{16}H_{15}NO_3Cl_2$ (340.22): C, 56.49; H, 4.44; Cl, 20.84. Found: C, 55.91; H, 4.76; Cl, 20.76.

N^{α}-Boc-*O*-(2,6-dichlorobenzyl)tyrosine. *O*-(2,6-Dichlorobenzyl)-tyrosine (28 g, 82 mmol) was converted to the N^{α}-Boc derivative by standard procedures.[34] The product crystallized from ether–petroleum ether at room temperature: 26 g (72%); mp 108–110°; tlc (CM) R_f 0.75; $[\alpha]^{24}D$ +21° (*c* 2, absolute ethanol).

Anal. Calcd for $C_{21}H_{23}NO_5Cl_2$ (440.34): C, 57.28; H, 5.27; Cl, 16.10. Found: C, 58.04; H, 5.75; Cl, 15.75.

The crystalline dicyclohexylamine salt gave mp 108–116°; $[\alpha]^{24}D$ +36.6° (*c* 2.2, $CHCl_3$). *Anal.* Calcd for $C_{33}H_{46}N_2O_5Cl_2$ (621.66): C, 63.76; H, 7.46; N, 4.51. Found: C, 63.61; H, 7.60; N, 4.95.

Boc-phenylalanyl Polymer. Boc-phenylalanine (2.69 g, 10.1 mmol) was dissolved in methanol (10 ml) and mixed with 15.5 ml of 0.62 *N* tetramethylammonium hydroxide in methanol. The solution was evaporated *in vacuo* to an oil which was reevaporated twice from dioxane and finally from methanol. After drying *in vacuo* over P_2O_5 for 1.5 hr the salt (3.51 g) was dissolved in DMF (50 ml) and stirred with 5.02 g of chloromethylated polymer for 14 hr at room temperature. The resin was filtered off and washed with DMF (200 ml), methanol (100 ml), water (100 ml), and methanol (100 ml). The product was dried *in vacuo* over P_2O_5 overnight to give 5.60 g. A sample after deprotection and neutralization gave the following amine content: 0.42 mmol/g by the test of Esko, *et al.*,[36] 0.43 mmol/g by the Gisin test,[37] and no visible trace of yellow color persisted on the resin after completion of the test.

For determination of the optical purity of the esterified phenylalanine, a sample (100 mg) of the above product was carried through standard procedures to prepare N^{α}-Boc-γ-Bzl(*p*-Br)Glu-Phe polymer.[38] The product was treated with HF (10 ml) for 30 min at 0° in the presence of anisole (0.5 ml). After removal of HF and drying, the resin was extracted with 10 ml of 50% TFA in CH_2Cl_2 and filtered off. The filtrate was evaporated to dryness. Chromatography performed according to the procedure of Manning and Moore[14] gave one major peak, L-Glu-L-Phe, following phenylalanine, and an extremely small peak close to the position of L-Glu-D-Phe represented 0.2% of the material in the major peak. An LAP digest of the dipeptide followed by tlc (BAW) gave spots corresponding to glutamic acid (R_f 0.22) and phenylalanine (R_f 0.50) and no spot corresponding to dipeptide (R_f 0.45).

N^{α}-Boc-γ-Bzl-Glu-Phe Polymer by Limited Coupling. Boc-phenylalanyl polymer (4.1 g) prepared as described above was treated as follows at 24° unless otherwise specified: 2 × 60 ml of CH_2Cl_2 (retention volume of resin was 23 ml); 42 ml of TFA and 18 ml of CH_2Cl_2, 17 min; 6 × 60 ml of CH_2Cl_2; 60 ml of 10% diisopropylethylamine in CH_2Cl_2, 7 min; 6 × 60 ml of CH_2Cl_2; at 4° 1.4 mmol of N^{α}-Boc-γ-Bzl-Glu in 60 ml of CH_2Cl_2, 10 min; at 4° 1.4 mmol of DCC in 5 ml of CH_2Cl_2, 90 min; at 4° 3 × 60 ml of CH_2Cl_2; 60 ml of CH_2Cl_2; 60 ml of 10% acetic anhydride in CH_2Cl_2, 10 min; 2 × 60 ml of CH_2Cl_2; 60 ml of 5% diisopropylethylamine in CH_2Cl_2, 5 min; 3 × 60 ml of CH_2Cl_2; 60 ml of 10% acetic anhydride in CH_2Cl_2, 5 min; 2 × 60 ml of CH_2Cl_2; 60 ml of 5% diisopropylethylamine in CH_2Cl_2, 5 min; 3 × 60 ml of CH_2Cl_2; 3 × 60 ml of absolute ethanol. The product was dried *in vacuo* over P_2O_5 overnight: 4.20 g. A sample subjected to the Gisin test[37] for amine content gave 0.25 μmol/g. A sample was refluxed in propionic acid–concentrated HCl[39] (1:1) for 18 hr, and amino acid analysis[35] gave 0.29 mmol of glutamic acid and 0.44 mmol of phenylalanine per gram of resin.

Protected Peptide Resin of α_h-ACTH. N^{α}-Boc-γ-Bzl-Glu-Phe polymer (3.23 g) prepared as just described was placed in a Beckman Model 990 peptide synthesizer and carried through the schedule shown in Tables IV and V for the introduction of each of the remaining 37 amino acid residues. N^{α}-Boc protection was used throughout with the following side-chain protecting groups: Lys, Z(*o*-Br)[17]; His, Boc[15]; Arg, tosyl[15]; Asp, Bzl; Ser, Bzl; Glu, Bzl; Tyr, Bzl(2,6-Cl_2).[17] The additional double coupling program was used for each valine residue. The active ester program was used for the asparagine residue. In the Lys-21 cycle step 7 was changed to 10% DIA in CH_2Cl_2 and used in this step thereafter. At the end of the Val-20 cycle the resin was thoroughly dried *in vacuo* over P_2O_5 and a 20% aliquot was removed. In the arginine-8 cycle (deprotection of tryptophan-9) steps 2 and 3 were deleted and the following steps were performed in the manual mode: 40 ml of 25% TFA in CH_2Cl_2, 1 min; 40 ml of 25% TFA in CH_2Cl_2, 30 min; 40 ml of 55% TFA in CH_2Cl_2, 2 min; 40 ml of 55% TFA in CH_2Cl_2, 4 min. In the arginine-8 cycle 1,2-ethanedithiol was added to the solvent in step 5 to a concentration of 0.25% (v/v) and used in this step thereafter. The finished peptide resin was dried *in vacuo* over P_2O_5 overnight to yield 5.61 g.

α_h-ACTH. Peptide resin (1.0 g) was treated with HF (16 ml) for 1 hr at 0° in the presence of anisole (2 ml). After removal of HF and thorough drying *in vacuo*, the resin was extracted with TFA (15 ml) and filtered off. The filtrate was evaporated *in vacuo* to an oil which was reevaporated from HOAc three times. The resulting product was stirred in water (20 ml) and washed with two 20-ml portions of ether. The aqueous solution was placed on a 55-ml column of Dowex 1-X4 (50–100 mesh, acetate form in water) and eluted with water. The first 108 ml of eluate was collected and lyophilized. The product was dissolved in 1 *N* acetic acid (5 ml) and subjected to gel filtration on a 2.2 × 25 cm Sephadex G-10 column in 1 *N* HOAc with collection of 3-ml fractions. Spectrophotometric measurements at 280 nm gave one peak (fractions 12–18) which after lyophilization gave 346 mg. This product was subjected to

(34) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).

(35) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(36) K. Esko, S. Karlsson, and J. Porath, *Acta Chem. Scand.*, **22**, 3342 (1968).

(37) See B. F. Gisin, ref 12.

(38) The preparation and use of N^{α}-Boc-γ-Bzl(*p*-Br)glutamic acid will be described elsewhere: D. Yamashiro, R. L. Noble, and C. H. Li, manuscript in preparation.

(39) J. Scotchler, R. Lozier, and A. B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).

Table IV. Schedule for DCC Coupling in Solid-Phase Synthesis of α_h-ACTH

DCC coupling	Step (program line)	Reagents and operations[a]	Mix times, min
Single	1	CH_2Cl_2 wash 40 ml (4 times)	2
	2	55% TFA in CH_2Cl_2 40 ml (1 time)	2
	3	55% TFA in CH_2Cl_2 40 ml (1 time)	15
	4	CH_2Cl_2 wash 40 ml (3 times)	2
	5	33% dioxane in CH_2Cl_2 40 ml (3 times)	2
	6	CH_2Cl_2 wash 40 ml (3 times)	2
	7	5% DIA in CH_2Cl_2 40 ml (1 time)	5
	8	CH_2Cl_2 wash 40 ml (1 time)	2
	9	CH_2Cl_2 wash 40 ml (5 times)	2
	10	Boc-AA (3.8 mmol) in 20 ml of CH_2Cl_2 (1 time)[b]	
	11	Boc-AA, check, transfer, and mix	10
	12	DCC (3.6 mmol) in 10 ml with CH_2Cl_2 (1 time)	2
	13	CH_2Cl_2 rinse 5 ml (1 time)	
	14	CH_2Cl_2 metering vessel rinse held 40 ml and coupling time (1 time)	150
	15	CH_2Cl_2 wash 40 ml (3 times)	2
	16	Abs EtOH wash 40 ml (3 times)	2
Double	Same as lines 9–16 in above program except that line 9 was executed 4 times		

[a] AA, aminc acid. [b] Boc(Tos)Arg and Boc-Trp were dissolved in 10% DMF in CH_2Cl_2.

Table V. Schedule for Active Ester Coupling in Solid-Phase Synthesis of α_h-ACTH[a]

Step (program line)	Reagents and operations[b]	Mix times, min
9	CH_2Cl_2 wash 40 ml (2 times)	2
10	DMF wash 40 ml (3 times)	2
11	Boc-AA-ONP (9.0 mmol) in 20 ml of DMF (1 time)	
12	Boc-AA, check, and transfer	
13	DMF rinse 5 ml (1 time)	
14	CH_2Cl_2 metering vessel rinse held 5 ml (1 time)	
15	Coupling time (4 times)	150
16	DMF wash 40 ml (3 times)	2
17	Abs EtOH wash 40 ml (3 times)	2

[a] Same as lines 1–8 in Table IV followed by the changes shown. [b] AA, amino acid.

gel filtration on a 2.5 × 133 cm Sephadex G-25 column with collection of a 175-ml forerun and then 11-ml fractions. Two peaks were detected (280 nm), a small one at 260 ml and a large one at 400 ml. Isolation of the material represented by the large peak gave 202 mg. This material was dissolved in 0.1 *N* HOAc (6 ml) along with dithiothreitol (101 mg) and heated at 50° for 21 hr.[20] The solution was applied to a carboxymethylcellulose column and chromatographed as shown in Figure 2. Isolation of material represented by the major peak (490–600 ml) gave 68 mg. A sample (20 mg) of this material was rechromatographed on CMC as shown in Figure 3; the peak emerged at 537 ml. When natural α_h-ACTH[23] (25 mg) was chromatographed under identical conditions a peak at 540 ml was obtained. Another sample (32 mg) of partially purified synthetic material from the first CMC chromatography was then subjected to partition chromatography on a 2.56 × 46 cm Sephadex G-50 column with collection of 7.1-ml fractions. When natural α_h-ACTH (5 mg) is chromatographed under these conditions on a 1.89 × 38 cm column with collection of 3.55-ml fractions a peak with R_f 0.28 is obtained (Figure 4). The synthetic material gave a pattern shown in Figure 5 with a major peak at R_f 0.26. Isolation of material represented by this peak (tubes 30–38) gave 12 mg. This material after gel filtration on a 1.38 × 114 cm Sephadex G-25 column in 0.5 *N* HOAc gave 11 mg of highly purified synthetic α_h-ACTH (peptide content 76% by ultraviolet absorption spectra; 3% yield based on Boc-Glu(Bzl)Phe-resin).

A sample (3 mg) of highly purified synthetic α_h-ACTH was again subjected to partition chromatography on a 1.89 × 38 cm Sephadex G-50 column with collection of 3.55-ml fractions as shown in Figure 6. A single symmetrical peak with R_f 0.27 was obtained in very close agreement with the value for natural hormone. On paper electrophoresis in pyridine acetate buffer (pH 3.7, 400 V, 4 hr, Whatman 3 MM) the synthetic material (200 μg) and natural hormone (200 μg) each gave a single ninhydrin spot with R_f 0.55 relative to lysine (Figure 7). Electrophoresis of synthetic material and natural hormone on polyacrylamide gel at pH 4.5 showed identical behavior (Figure 8). Amino acid analyses[35] of an acid hydrolysate of synthetic material gave the results shown in Table I.

Samples (0.35 mg) of synthetic material were treated separately with 7 μg each of trypsin and chymotrypsin in 35 μl of Tris buffer (pH 8.5, 0.01 *M* Mg^{2+}) at 37° for 6 hr. Samples of natural α_h-ACTH were also treated in an identical manner. Paper electrophoresis of the digests (*ca.* 15-μl aliquots) in collidine acetate buffer (pH 6.9, 400 V, 4 hr) gave the peptide patterns (ninhydrin detection) shown in Figure 9.

Optical rotatory dispersion and circular dichroism spectra of synthetic and natural hormones were measured in 0.1 *N* acetic acid in a Cary 60 recording spectropolarimeter equipped with a Model 6002 circular dichroism attachment. The general procedures used in this laboratory for these measurements have been described.[40,41] Circular dichroism spectra were obtained on solutions of synthetic and natural hormones at concentrations of 1.104 and 1.215 mg/ml, respectively, at 27° in a 1.0-cm path length cell for wavelengths above 250 nm and in a 1.0 mm cell below 250 nm. Optical rotatory dispersion measurements were obtained on the same solutions under the aforementioned conditions except for the value at 208 nm where the solutions were diluted threefold. In all measurements dynode voltages did not exceed 500 V. Peptide concentrations were then determined on the diluted solutions from their absorption spectra taken from 360 to 240 nm on a Beckman DK-2A recording spectrophotometer with correction for light scattering[42] and with the assumption that $E_{276\,nm}^{1\%,\,1cm} = 17.7$. The two samples showed identical absorption spectra in the region just mentioned.

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(40) T. A. Bewley, J. Brovetto-Cruz, and C. H. Li, *Biochemistry*, **8**, 4701 (1969).
(41) T. A. Bewley and C. H. Li, *Biochim. Biophys. Acta*, 140, 201 (1967).
(42) See Beavan and Holiday, Table I, footnote *b*.