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Studies on Polypeptides. XLV. Structure–Function Studies in the β-Corticotropin Series[1-4]

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Two analogs of β-corticotropin$_{1-20}$ amide, *i.e.*, glutamine^5-β-(pyrazolyl-3)-alanine^6-β-corticotropin$_{1-20}$ amide and glutamine^5-phenylalanine^9-β-corticotropin$_{1-20}$ amide were synthesized and certain biological properties of these peptides were evaluated. The observation that the replacement of histidine by β-(pyrazolyl-3)-alanine only decreased but did not eliminate steroidogenic and melanocyte expanding activities demonstrates conclusively that these biological properties of β-corticotropin are not dependent on the characteristic acid–base behavior of the imidazole portion of histidine. Thus, intravenous infusion of the pyrazole analog raised the plasma 11-hydroxycorticosteroid level and the urinary 17-keto steroid:creatinine ratio in human subjects. The same dose administered intramuscularly by a single injection failed to elicit these effects. Glutamine^5-phenylalanine^9-β-corticotropin$_{1-20}$ amide exhibits only 2–3% *in vivo* steroidogenic and adrenal ascorbic acid depleting activity of β-corticotropin on a weight basis The *in vitro* melanocyte expanding potency of this material was 1.4×10^7 MSH units/g. It is concluded that the indole portion of tryptophan is not functionally involved in the physiological activity of β-corticotropin but appears to contribute to the binding of the hormone to its receptor(s). Certain structure–function relations in the β-corticotropin series are discussed.

Structure–function studies with homogeneous synthetic fragments of β-corticotropin (I) (Table I) have corroborated the initial observation of Bell, *et al.*,[5] that the biologically effective unit of this hormone is located in the N-terminal region between positions 1 and 24. β-Corticotropin$_{1-24}$ (III),[6] β-corticotropin$_{1-23}$ (X),[7] β-corticotropin$_{1-23}$ amide (XI),[8] and β-corticotropin$_{1-20}$ amide (XVI)[9] exhibit approximately the same *in vivo* adrenocorticotropic activity in the rat as natural β-corticotropin.[7,10-12]

Methods developed for the synthesis of these compounds have been applied to the preparation of analogs whose biologic evaluation may pinpoint functionally essential amino acid residues.

In the present investigation we explored the importance for *in vivo* adrenocorticotropic and *in vitro* melanocyte expanding activity of the histidine and tryptophan residues.

In previous communications[13-16] we have described the synthesis of the unnatural amino acid L-β-(pyrazolyl-3)-alanine[Pyr(3)Ala] and have discussed its value as a tool to assess the importance for biological activity of the acid–base properties of the imidazole ring in histidine. The significance for adrenocorticotropic and melanocyte-expanding activity of the characteristic imidazole dissociation of histidine has now been evaluated with synthetic glutamine^5-β-(pyrazolyl-3)-alanine^6-β-corticotropin$_{1-20}$ amide (XVIII).

The finding of Dedman, *et al.*,[17] that treatment of β-corticotropin with *p*-dimethylaminobenzaldehyde in acid solution resulted in loss of biological activity was interpreted by these authors "to indicate that the 2 position of the tryptophan residue is probably essential for biological activity, although the possibility of side reactions cannot be excluded."

(1) See *J. Amer. Chem. Soc.*, in press (1970), for paper XLIV in this series.

(2) Supported by grants from the U. S. Public Health Service and the Hoffmann-La Roche Foundation.

(3) A preliminary communication of some of the results presented in this study has appeared: K. Hofmann, H. Bohn, and R. Andreatta, *J. Amer. Chem. Soc.*, **89**, 7126 (1967).

(4) The amino acid residues except glycine are of the L-configuration. Abbreviations used are: Boc = *t*-butoxycarbonyl; Z = benzyloxycarbonyl; ONHS = *N*-hydroxysuccinimido; Pyr(3)ala = β-(pyrazolyl-3)-alanine; TEA = triethylamine; TFA = trifluoroacetic acid; CMC = carboxymethylcellulose; AP-M = aminopeptidase M [G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)].

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TABLE I

STRUCTURES AND BIOLOGICAL ACTIVITIES OF β-CORTICOTROPIN AND ANALOGS

PEPTIDE NO	AMINO ACID SEQUENCE[a]	BIOLOGICAL ACTIVITY ACTH U/mg [b]	ACTH U/mg [c]	MSH U/g [d]
I	H-SER·TYR·SER·MET·GLU·HIS·PHE·ARG·TRP- - - - -VAL·LYS·VAL·TYR·PRO- - - - -PHE·OH (1 2 3 4 5 6 7 8 9 ... 20 21 22 23 24 ... 39)	100	100	10^8
II	H-SER-NLE- - - - -VAL-NH_2	625		
III	H- - - - -OH	100	100	10^8
IV	H-SER- - - - -OH	750	300	10^9
V	H-ALA- - - - -OH	500	300	10^{10}
VI	H-SER·TYR- - - - -OH		100	10^9
VII	H-SER·TYR·SER·MET- - - - -OH		100	10^9
VIII	H- - - - -GLU·HIS·PHE·ARG·TRP		0	10^6
IX	H- - - - -ORN- - - - -OH		1	10^7
X	H- - - - -OH	116	91	10^8
XI	H- - - - -NH_2	100		
XII	H-GLY- - - - -NH_2	104		
XIII	H-PHE- - - - -NH_2	66		
XIV	H-ALA- - - - -NH_2	50		
XV	H- - - - -NH_2	51		
XVI	H- - - - -NH_2	111	83	10^8
XVII	H-ABUT·GLN- - - - -NH_2	31	74	10^7
XVIII	H-GLN·PYR- - - - -NH_2	50	60	10^7
XIX	H-GLN- - - - -PHE- - - - -NH_2	2	2	10^7

[a] See text for lit. refs to the various compounds. Amino acid residues shown in italics are of the D-configuration. [b] Adrenal ascorbic acid depleting activity. [c] *In vivo* rat steroidogenic activity. [d] *In vitro* melanocyte expanding activity.

In order to derive more information pertaining to the importance of the tryptophan residue for hormonal activity we prepared glutamine[5]-phenylalanine[9]-β-corticotropin$_{1-20}$ amide (XIX) and evaluated its *in vivo* adrenocorticotropic and *in vitro* melanocyte expanding potencies.

Preparative Aspects.—The key reactions in the synthesis of XVIII and XIX (Table I) are illustrated in Scheme I. Histidylphenylalanylarginylphenylalanylglycine (XX) was prepared by the stepwise method from methyl phenylalanylglycinate. A mixed anhydride of benzyloxycarbonylnitroarginine[18] served to introduce the arginine residue. The ensuing methyl benzyloxycarbonylnitroarginylphenylalanylglycinate was saponified and the benzyloxycarbonyltripeptide converted into arginylphenylalanylglycine by catalytic hydrogenation. Routine elongation of the chain with *N*-hydroxysuccinimidobenzyloxycarbonylphenylalaninate[19] and benzyloxycarbonylhistidine azide[20] afforded XX.

For the synthesis of the protected decapeptide XXII the pentapeptide XX was treated with the azide XXI. Adsorption on Dowex-50 and elution with aqueous MeOH containing TEA provided a suitable procedure for isolation of XXII from the reaction mixture.

The protected decapeptide XXV, a key intermediate for the preparation of XVIII, resulted from the coupling of XXIV[14] with the azide XXI; it was isolated as described for the preparation of XXII. The tosylate salt of XXV was reacted with the tosylate salt of XXIII using DCI as the condensing reagent.[6,21] Following exchange of tosylate for acetate ions the mixture of products was subjected to gel filtration on Sephadex G 25[8] for isolation of homogeneous *N*-*t*-butoxycarbonylseryltyrosylserylmethionylglutaminyl-β-(pyrazolyl-3)-alanylphenylalanylarginyltryptophylglycyl-N^{ϵ}-*t*-butoxycarbonyllysylprolylglycyl-N^{ϵ}-*t*-butoxycarbonyllysyl-N^{ϵ}-*t*-butoxycarbonyllysylarginylarginylprolylvaline amide acetate. This compound was deblocked with 90% TFA, trifluoroacetate ions were exchanged for acetate ions, and the ensuing eicosapeptide amide acetate XVIII was purified by cmc chromatography.

The synthesis of XIX involved coupling by the DCI

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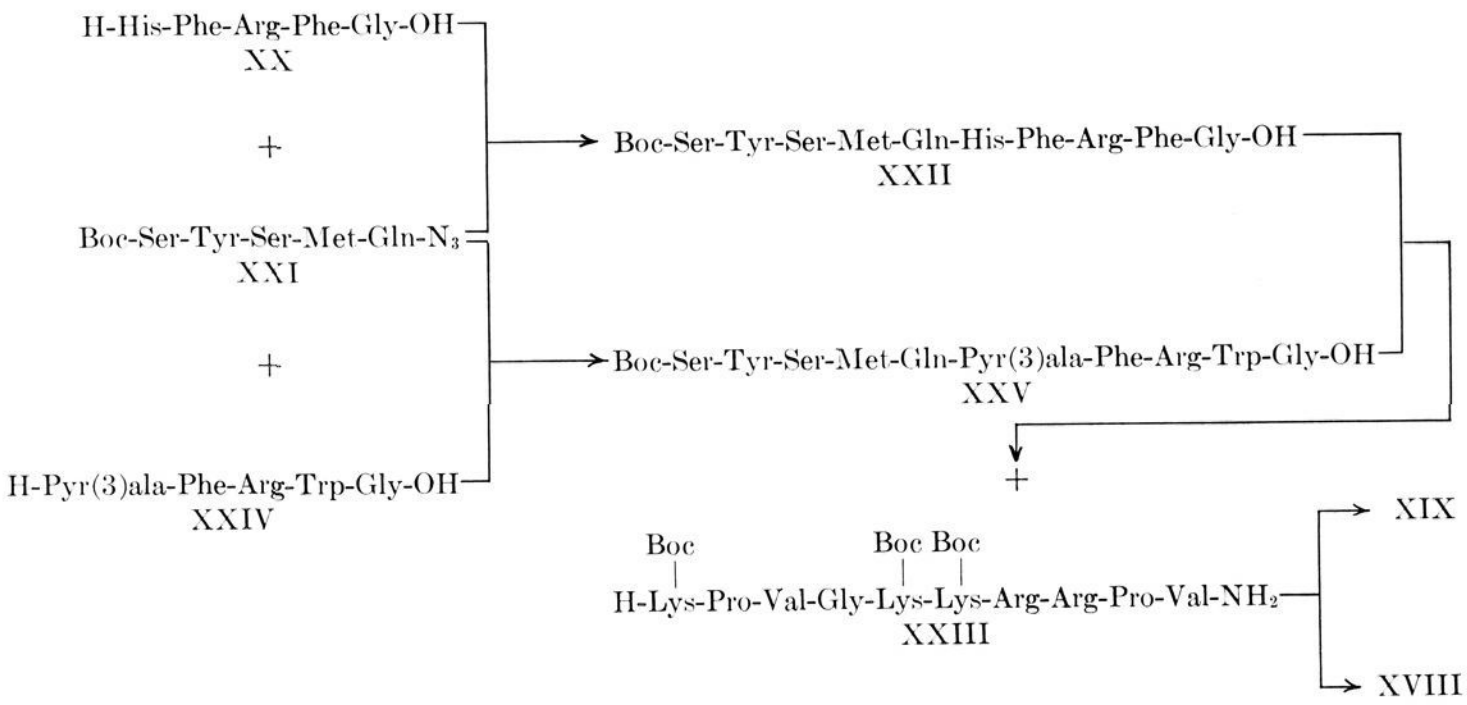
Scheme I



procedure of the tosylate salt XXII and XXIII[21] in pyridine–DMF followed by conversion of the reaction products into the acetate salts. Since attempts to isolate the desired protected eicosapeptide amide by gel filtration were unsuccessful, presumably due to a difference in aromatic interaction of Trp and Phe with the Sephadex, the reaction products were deblocked with TFA and XIX was isolated, in poor yield, by CM-cellulose chromatography followed by desalting on Bio-Rex 70.

The eicosapeptide amides XVIII and XIX were homogeneous as judged by tlc and disc electrophoresis (Figure 1) and both compounds were completely digestible by AP-M. The constituent amino acids were present in the digests in the ratios predicted by theory.

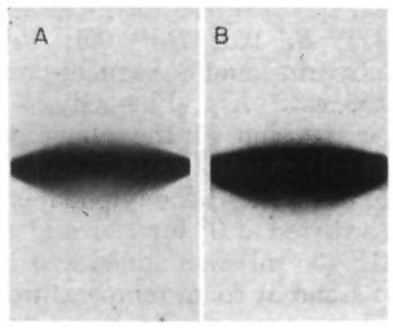


Figure 1.—Disc electropherograms of compound XVIII, A and compound XIX, B. See text for experimental conditions.

Experimental Section

A. General Procedures.—Melting points are uncorrected. Rotations were determined with a Zeiss precision polarimeter. Measurements were carried out with a Hg lamp at 546 and 578 mμ and extrapolated to the 589-mμ Na line. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.; *O values were actually determined and not* computed by difference. Disc electropherograms were stained with aniline blue–black in 7.5% AcOH. They were performed with 25 μg of peptide in 7.5% polyacrylamide gel at a running pH of 3.8. The amino acid composition of acid and enzymic hydrolysates was determined with a Beckmann-Spinco Model 120 amino acid analyzer.[22] The figures in parentheses are average recoveries of amino acids based on formula weight. Nle and α-amino-β-guanidopropionic acid were used as internal standards. Acid hydrolysates were performed in constant boiling HCl at 110° for 24 hr in evacuated tubes; values are not corrected for amino acid destruction. AP-M digests and thioglycolate reductions were carried out as described.[23] See Hofmann, *et al.*,[24] for designation of solvent systems for paper and tlc chromatograms.

The rat adrenal ascorbic acid depleting assay was performed according to the U. S. Pharmacopeia.[25] Steroidogenic activity was determined in hypophysectomized or dexamethasone-blocked rats.[26a,b] The method of Shizume, *et al.*,[27] served to determine *in vitro* melanocyte expanding potency.

B. Preparation of Peptides. Methyl Benzyloxycarbonylphenylalanylglycinate.—*N*-Hydroxysuccinimido benzyloxycarbonylphenylalaninate[19] (19.9 g) and methyl glycinate·HCl (6.28 g) were suspended in THF (300 ml) and the suspension was cooled at 0°. TEA (14 ml) was added and the mixture was kept at 0° for 1 hr and at room temperature for 24 hr. The solvent was removed, the residue was dissolved in EtOAc (400 ml), and the solution was washed in the usual manner and dried. Evaporation of the solvent gave an oil which soon crystallized. The material was recrystallized from EtOAc–petroleum ether: 17.4 g (94%); mp 118–119°; $[\alpha]^{26}D$ −17.2° (*c* 2.09, MeOH); $[\alpha]^{29}D$ −22.7° (*c* 1.4, DMF); R_f^{I} 0.8; R_f^{III} 0.8; R_f^{IV} 0.9. lit.[28] mp 122–123°; $[\alpha]^{20}D$ −23.6° (*c* 2.0, DMF). *Anal.* ($C_{20}H_{22}N_2O_5$) C,H,N.

Methyl Phenylalanylglycinate Hydrochloride Hemihydrate.—The benzyloxycarbonyl derivative (11.1 g) was hydrogenated over Pd in a mixture of MeOH (100 ml) and 1 *N* HCl (30 ml). The catalyst was removed by filtration, the filtrate was evaporated, and the residue was lyophilized from dioxane; yield quantitative; $[\alpha]^{25}D$ +52.3° (*c* 1.8, H_2O); R_f^{I} 0.6; R_f^{III} 0.8; single ninhydrin- and Cl-positive spot. *Anal.* ($C_{12}H_{16}N_2O_3 \cdot HCl \cdot 0.5\ H_2O$) C, H, N.

Benzyloxycarbonylnitroarginylphenylalanylglycine.—A mixed anhydride prepared from benzyloxycarbonylnitroarginine (3.54 g), *n*-Bu_3N (2.4 ml), and ethyl chloroformate (0.96 ml) in dioxane (70 ml) was added to a solution of methyl phenylalanylglycinate·HCl (2.72 g) in dioxane (50 ml) containing TEA (1.4 ml) and the reaction mixture was allowed to stand at room temperature for 2 hr. The solvent was evaporated and the residue was

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distributed between H_2O and EtOAc–*n*-BuOH (6:4). The organic phase was washed in the usual manner and the bulk of the solvents evaporated. Et_2O (400 ml) was added to the remaining mass, and the precipitate was collected, washed (Et_2O), and dried: 4.54 g (80%); mp 155–156°; $[\alpha]^{25}D$ −24.2° (*c* 1.94, MeOH); R_f^I 0.8; R_f^{III} 0.8; R_f^{IV} 0.9; R_f^S 3.7 × His; single Cl-positive spot. For saponification the Me ester (14.15 g) was suspended in dioxane (100 ml) and 1 *N* NaOH (30 ml) was added with stirring. After 15 min a clear solution was obtained, then the Na salt of the desired product started to precipitate as white needles. The suspension was stirred at room temperature for 2 hr, dioxane (200 ml) was added, and the mixture was placed in a refrigerator. The solid was collected, washed with dioxane, and dissolved in H_2O (200 ml). The desired compound precipitated upon addition of 1 *N* HCl: 13.27 g (96%); mp 227–230° dec. A small sample was reprecipitated from 80% AcOH with H_2O: mp 231–233° dec; $[\alpha]^{25}D$ −17.4° (*c* 2.15, DMF); R_f^I 0.7; R_f^{III} 0.7; R_f^{IV} 0.4; R_f^V 0.8; R_f^S 4.1 × His; single Cl-positive spot. *Anal.* ($C_{25}H_{31}N_5O_5$) C, H, N, O.

Benzyloxycarbonylphenylalanylarginylphenylalanylglycine Monohydrate.—Benzyloxycarbonylnitroarginylphenylalanylglycine (12.8 g) was hydrogenated for 24 hr over Pd in MeOH (200 ml) containing 10% AcOH (50 ml). Fresh catalyst was added after 8 hr of hydrogenation. The catalyst was removed by filtration, the filtrate was concentrated to a small volume, and the residue was lyophilized to constant weight from small volumes of H_2O: yield quantitative; hygroscopic powder; $[\alpha]^{25}D$ +2.5° (*c* 2.66, H_2O)); R_f^I 0.3; R_f^{III} 0.5; R_f^I 0.4; R_f^S 2.6 × His; single Cl-, ninhydrin-, and Sakaguchi-positive spot; amino acid ratios in AP-M digest: $Arg_{1.0}Phe_{1.0}Gly_{1.0}$.

To an ice-cold suspension of the above tripeptide acetate (4.4 g) in THF (120 ml) containing TEA (4.1 ml) was added *N*-hydroxysuccinimido benzyloxycarbonylphenylalaninate[19] (3.96 g) and the mixture was stirred at 0° for 1 hr and at room temperature for 1 hr. DMF (35 ml) was added and the resulting solution was allowed to stand at room temperature for another 2 hr. The bulk of the solvents was removed *in vacuo* and H_2O (300 ml) was added to the residue. After cooling in a refrigerator, the precipitate was collected, washed with ice-water, and dried. The product was reprecipitated from 90% AcOH with H_2O: 4.96 g (73%); mp 251–253° dec; $[\alpha]^{29}D$ −10.6° (*c* 1.53, 90% AcOH); R_f^I 0.5; R_f^{III} 0.7; R_f^S 3.7 × His; single Cl- and Sakaguchi-positive spot; amino acid ratios in acid hydrolysate: $Phe_{2.0}Arg_{1.0}Gly_{1.0}$. *Anal.* ($C_{34}H_{41}N_7O_7 \cdot H_2O$) C, H, N; O calcd 18.9, found 19.5.

Phenylalanylarginylphenylalanylglycine Monoacetate.—A suspension of the benzyloxycarbonyl derivative (4.55 g) in MeOH–20% AcOH (1:1) (10 ml) was hydrogenated over Pd. The catalyst was removed by filtration, the filtrate was concentrated to a small volume, and the residue was lyophilized: white powder; 3.9 g (99%); $[\alpha]^{26}D$ +4.1° (*c* 1.07, H_2O)); R_f^I 0.3; R_f^{III} 0.6; single Cl- and ninhydrin-positive spot; amino acid ratios in AP-M digest: $Phe_{2.0}Arg_{1.0}Gly_{1.0}$. *Anal.* ($C_{26}H_{35}N_7O_5 \cdot CH_3CO_2H$) C, H, N, O.

Benzyloxycarbonylhistidylphenylalanylarginylphenylalanylglycine Monoacetate Monohydrate.—To a solution of benzyloxycarbonylhistidine hydrazide[20] (3.03 g) in DMF (30 ml) was added at −30° 6.9 *N* HCl in dioxane (5 ml) followed by *t*-butyl nitrite (1.3 ml). The solution was kept at −30° for 30 min and then cooled at −60°. The pH was adjusted to 8.0–8.5 by addition of TEA (approximately 5 ml) and a solution of phenylalanylarginylphenylalanylglycine acetate (2.94 g) in DMF (20 ml) containing TEA (0.8 ml) was added over a period of 15 min. The temperature was allowed to reach 4°, and the mixture was stirred for 24 hr (pH maintained at 8.0–8.5 by addition of TEA). The bulk of the solvent was evaporated, EtOAc (200 ml) was added to the residue and the mixture was placed in a refrigerator for 2 hr. The solid was collected, washed with EtOAc, and dried. The residue was triturated with H_2O (150 ml), the ensuing suspension was placed in a refrigerator for 1 hr, and the solid was collected, washed with five 20-ml portions of ice-water, and dried. Yield of crude material (hydrochloride), which was used for the next step without purification was 3.95 g (90%). A sample for analysis (85 mg) was dissolved in 50% aqueous EtOH (30 ml) and the solution was passed through a column of Amberlite IRA-400 (2 × 6 cm). Cl-positive eluates were evaporated to a small volume and lyophilized from H_2O–dioxane: 76 mg; $[\alpha]^{25}D$ −21.4° (*c* 1.63, DMF); R_f^I 0.5; R_f^{III} 0.7; single Cl- and Pauly-positive spot; amino acid ratios in acid hydrolysate: $His_{1.2}Phe_{0.8}Arg_{0.9}Gly_{1.0}$. *Anal.* ($C_{36}H_{45}N_{10}O_7 \cdot CH_3CO_2H \cdot H_2O$) C, H, N, O.

Histidylphenylalanylarginylphenylalanylglycine Sesquihydrate (XX).—The protected pentapeptide (3.1 g) was hydrogenated over Pd in 50% AcOH. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in H_2O (150 ml), the solution was cooled at 0° and Dowex 50W-X2 (200–400 mesh) (10 g) was added. The suspension was stirred at 0° for 45 min; the resin was collected and washed with ice-cold H_2O. The filtrate was treated with fresh resin (5 g) in the same manner and the combined resins were suspended in ice-water (150 ml). TEA was added dropwise with stirring until the pH reached 8–8.5, and the suspension was stirred for 10 min. The resin was removed by filtration, was resuspended in ice-water (50 ml) containing TEA and the suspension was stirred for 10 min and filtered. The filtrate and washings were concentrated to a small volume and lyophilized: 1.55 g, (63%); $[\alpha]^{28}D$ −15.9° (*c* 2.04, H_2O)); R_f^I 0.3; R_f^{III} 0.5; single Cl-, Pauly-, and ninhydrin-positive spot; amino acid ratios in AP-M digest: $His_{1.0}Phe_{0.9}Arg_{0.9}Gly_{1.0}$. *Anal.* ($C_{26}H_{42}N_{10}O_6 \cdot 1.5\ H_2O$) C, H, N, O.

***N*-*t*-Butoxycarbonylseryltyrosylserylmethionylglutamine Monohydrate.**—*N*-*t*-Butoxycarbonylseryltyrosine hydrazide monohydrate[21] (4.3 g) was dissolved in 1 *N* HCl (45 ml) precooled at −5° and the solution was layered with ice-cold EtOAc (30 ml). $NaNO_2$ (5 *N*) was added dropwise with stirring until the solution turned KI–starch paper blue. After 5 min at −5° EtOAc (50 ml) was added and the two layers were separated. The aqueous phase was reextracted with two 50-ml portions of ice-cold EtOAc and the combined organic phases were washed with ice-cold saturated $NaHCO_3$ and H_2O and dried over $MgSO_4$ at −10°.

Ice-cold DMF (7 ml) was added, and the bulk of the EtOAc was removed *in vacuo* at 0°. The DMF solution was then added with stirring to an ice-cold solution of serylmethionylglutamine monohydrate[22] (2.05 g) in H_2O (5 ml) and TEA (0.85 ml). The reaction mixture was kept at 5° for 3 days, the pH being maintained at approximately 8 by addition of TEA. NH_4OH, 3% (80 ml), was added and the solution was extracted with several portions of EtOAc until contaminating tyrosine-positive, methionine-negative material was removed. The organic phases were reextracted with 3% NH_4OH and the combined aqueous layers were adjusted to pH 7 with AcOH. The solution was concentrated to a small volume, the resulting gelatinous mass was extracted into *n*-BuOH (150 ml), and the solution was washed with six 50-ml portions of 10% AcOH and three 50-ml portions of H_2O. The *n*-BuOH layers were evaporated, and the product was precipitated twice from *i*-PrOH with EtOAc: 3.06 g (78%); mp 147–148° dec.; R_f^I 0.6; R_f^{III} 0.7; single Cl-positive, ninhydrin-negative spot; $[\alpha]^{25}D$ −28.0° (*c* 1.05 MeOH). *Anal.* ($C_{30}H_{46}N_8O_{12}S \cdot H_2O$) C, H, N.

A 15-mg sample of the protected pentapeptide was dissolved in TFA (0.5 ml), the solution was allowed to stand at room temperature for 20 min, then the product was precipitated by addition of Et_2O (20 ml).

Trifluoroacetate ions were exchanged for acetate ions on Amberlite IRA-400, and the product was lyophilized: 11 mg; R_f^I 0.3; single Cl- and ninhydrin-positive spot; amino acid ratios in AP-M digest: $Ser_{2.0}Tyr_{1.0}Met_{0.9}Glu_{0.8}$ (85%).

***N*-*t*-Butoxycarbonylseryltyrosylserylmethionylglutamine Hydrazide Monohydrate.**—Ethereal CH_2N_2 was added to an ice-cold solution of *N*-*t*-butoxycarbonylseryltyrosylserylmethionylglutamine monohydrate (4.5 g) in MeOH (600 ml) until the solution remained yellow. After standing for 5 min the solvent was evaporated and the ensuing syrup was dissolved in MeOH (200 ml). Hydrazine hydrate (5 ml) was added and the mixture was kept at room temperature for 48 hr. The solution was placed in a refrigerator for 4 hr, the gelatinous precipitate was collected and dried over H_2SO_4. The material was precipitated from 90% (v/v) aqueous MeOH with EtOAc: 3.8 g (83%); mp 199–202° dec.; $[\alpha]^{28}D$ −6.8° (*c* 1.60, DMF); R_f^I 0.9; R_f^I 0.6; R_f^{III} 0.8; single Cl- and hydrazide-positive spot. *Anal.* ($C_{30}H_{51}N_9O_{10}S \cdot H_2O$) C, H, N, O.

***N*-*t*-Butoxycarbonylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginylphenylalanylglycine Trihydrate (XXII).**—*N*-*t*-Butoxycarbonylseryltyrosylserylmethionylglutamine hy-

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drazide monohydrate (747 mg) was dissolved with slight warming in a mixture of DMF (6 ml) and H_2O (0.5 ml). The solution was cooled at −10° and 1 *N* HCl (2.0 ml) was added followed by a volume of 10% aq $NaNO_2$ sufficient to turn KI–starch paper blue (approximately 0.75 ml). The reaction mixture was stirred vigorously at −10° for 10 min, then the pH was adjusted to 8.0 by addition of 10% TEA in DMF. A solution of XX (690 mg) in DMF (4 ml), H_2O (1 ml), and 10% TEA in DMF (1.5 ml) was added and the mixture was kept at 5° for 48 hr. Additional azide (prepared from 373 mg of hydrazide) was then added and the viscous mixture was stirred at 5° for 24 hr. The bulk of the solvents was removed, H_2O (100 ml) was added to the residue and the solution was extracted with four 100-ml portions of *n*-BuOH. The organic phases were washed (H_2O) and evaporated to dryness and the gelatinous residue was dissolved in 50% aq MeOH (100 ml). This solution was cooled at 0°, Dowex 50 W-X2 (30 ml settled in H_2O) was added, and the suspension was stirred at 0° for 45 min, then the resin was collected and washed by suspension with two 50-ml portions of ice-cold 50% MeOH The resin was resuspended in ice-cold 50% MeOH (100 ml), TEA was added dropwise with stirring until the pH of the supernatant reached 8–8.5, and the mixture was stirred at 0° for 30 min. The resin was collected and washed twice with ice-cold 50% MeOH containing TEA and the combined filtrates were concentrated to approximately 80 ml. The mixture was kept in a refrigerator for 2 hr and the precipitate was collected, washed with ice–water, and dried (482 mg). A sample for analysis was precipitated from DMF with H_2O: mp 212–214° dec; $[\alpha]^{26}D$ −24.0° (*c* 1.04, DMF); R_f^I 0.4; R_f^{III} 0.7; R_f^1 0.6; R_f^3 3.9 × His; single Cl-, methionine-, and Pauly-positive spot; amino acid ratios in acid hydrolysate; $Ser_{1.8}Tyr_{0.9}Met_{1.0}Glu_{1.1}His_{1.1}Phe_{2.0}Arg_{0.9}Gly_{1.0}$. *Anal.* ($C_{62}H_{86}N_{16}O_{17}S \cdot 3H_2O$) C, H, N, O.

Tosylate Salt.—The decapeptide trihydrate (XXII) (151 mg) was dissolved in 50% aqueous THF (16 ml), the solution was cooled to 0°, and a precooled solution of Ts monohydrate (41 mg) in H_2O (1 ml) was added. The solution was concentrated to approximately 8 ml at room temperature and was then lyophilized; 208 mg. Prior to its use the salt was dried for 1 hr *in vacuo* at 50°.

N-*t*-Butoxycarbonylseryltyrosylserylmethionylglutaminyl-β-(pyrazolyl-3)-alanylphenylalanylarginyltryptophylglycine Monohydrate (XXV).—To a solution containing the azide XXI (from 747 mg of the hydrazide as described above) was added a solution of XXIV[14] (500 mg) in DMF (3 ml) containing 1.25 ml of 10% TEA in DMF and the mixture was stirred at 5° for 24 hr. At this point a second portion of azide (from 250 mg of the hydrazide) was added and the gelatinous mass was stirred for an additional 48 hr at 5° (pH maintained at 7.5–8 by addition of TEA). The bulk of the solvents was removed and H_2O (250 ml) was added to the residue. The mixture was stored at 5° for 4 hr and the precipitate was collected and dried (P_2O_5). The ensuing white powder (850 mg) was dissolved in DMF (40 ml) and a mixture of H_2O–MeOH–*i*-PrOH (1:1:1) (60 ml) was added. The solution was cooled at 0°, Dowex 50 W-X2 (15 ml settled in the same solvent) was added, and the mixture was stirred for 1 hr at 0°. The resin was collected, washed with the same solvent, and resuspended in 150 ml of an ice-cold mixture of DMF–H_2O–MeOH–*i*-PrOH (2:1:1:1). TEA was added dropwise with stirring and cooling until the supernatant reached pH 8–8.5. The mixture was filtered and the resin was eluted twice more with 75 ml each of the same solvent containing a few drops of TEA, and the combined filtrates were concentrated to approximately 5 ml. The product was precipitated by addition of water (200 ml), the mixture was stored at 5° for 24 hr, then the product was collected and dried: 598 mg (62%); mp 200–203° dec; $[\alpha]^{27}D$ −15.4° (*c* 0.52, DMF); amino acid ratios in acid hydrolysate: $Ser_{1.9}Tyr_{0.9}Met_{1.0}Glu_{1.1}Pyr(3)ala_{1.0}Phe_{1.0}Arg_{1.2}Gly_{1.0}$; R_f^I 0.4; R_f^{III} 0.7; single chlorine- and Ehrlich-positive spot. *Anal.* ($C_{64}H_{87}N_{17}O_{13}S \cdot H_2O$) C, H, N, O.

For enzymic digestion a 13-mg sample was dissolved in TFA (0.75 ml), and the solution was allowed to stand at room temperature for 25 min. The solvent was evaporated and the residue lyophilized from H_2O containing a few drops of dioxane; R_f^I 0.4; R_f^{III} 0.6; single Cl- and Ehrlich-positive spot; amino acid ratios in AP-M digest: $(Ser + Glu)_{2.9}Tyr_{1.0}Met_{1.1}Pyr(3)ala_{1.0}Phe_{1.0}Arg_{1.0}Trp_{0.8}Gly_{1.1}$(Rec. calcd for di-TFA salt 85%).

Tosylate Salt.—The decapeptide monohydrate XXV (109 mg) was dissolved in 50% aqueous THF (50 ml) by slight warming. The solution was cooled at 0° and a precooled solution of Ts monohydrate (14 mg) in H_2O (1 ml) was added. The solution was concentrated to approximately 15 ml at room temperature and lyophilized. Prior to its use, the salt was dried *in vacuo* at 50° for 1 hr.

Tosylate Salt of N^ϵ-*t*-Butoxycarbonyllysylprolylvalylglycyl-N^ϵ-*t*-butoxycarbonyllysyl-N^ϵ-*t*-butoxycarbonyllysylarginylarginylprolylvaline Amide (XXIII).—Compound XXIII[21] (128 mg) was dissolved in H_2O (6 ml), the solution was cooled at 0°, and an ice-cold solution of Ts monohydrate (42 mg) in H_2O (2 ml) was added. The solution was lyophilized to give a colorless fluffy powder; 154 mg. Prior to its use the salt was dried *in vacuo* at 50° for 1 hr.

N-*t*-Butoxycarbonylseryltyrosylserylmethionylglutaminyl-β-(pyrazolyl-3)-alanylphenylalanylarginyltryptophylglycyl-N^ϵ-*t*-butoxycarbonyllysylprolylvalylglycyl-N^ϵ-*t*-butoxycarbonyllysyl-N^ϵ-*t*-butoxycarbonyllysylarginylarginylprolylvaline Amide Acetate Hydrate.—The dried tosylate salts derived from 109 mg of XXV and 128 mg of XXIII were dissolved in DMF (6 ml), and pyridine (3 ml) and DCI (30 mg) was added. The reaction mixture was allowed to stand at room temperature for 5 days and additional amounts of DCI (30 mg each) were added on the 2nd, 3rd, and 4th days. The solution was concentrated *in vacuo* to approximately 5 ml, EtOAc (150 ml) was added and the suspension was allowed to stand in a refrigerator for 2 hr, then the precipitate was collected and dried. This material was dissolved in 50% aqueous THF (100 ml), and the solution was passed through a column of Amberlite IRA-400 (1.2 × 15 cm) which was eluted with the same solvent. Cl-positive eluates were pooled, concentrated to approximately 10 ml, and insoluble material was removed by filtration through filter-cel. The filtrate was lyophilized to give a slightly yellow fluffy powder; 236 mg; tlc showed two spots with R_f^I 0.5; (Cl- and Ehrlich-positive) and 0.2 (Cl-positive, Ehrlich-negative). This material dissolved in 1% AcOH (4 ml) was applied to a column (1.8 × 228 cm) of Sephadex G 25 fine and the column was developed with 1% AcOH. Fractions (3 ml each) were collected at a flow rate of 1 ml/min. Absorbancy at 280 mμ and tlc located the desired material in fractions 147–162. These fractions were pooled,the bulk of the solvent was removed, and the residue lyophilized to give a fluffy colorless powder; 108 mg. Fractions 135–146 were, also, pooled and lyophilized. The ensuing material (110 mg) was rechromatographed giving an additional quantity (46 mg) of homogeneous protected eicosapeptide: $[\alpha]^{27}D$ −50.4° (*c* 0.93, 10% AcOH; R_f^I 0.5; R_f^{III} 0.7; amino acid ratios in acid hydrolysate: $Ser_{2.1}Tyr_{0.9}Met_{1.1}Glu_{2.0}Pyr(3)ala_{1.0}Phe_{1.0}Arg_{2.9}Gly_{2.0}Lys_{3.0}Pro_{2.0}Val_{2.1}$.

Seryltyrosylserylmethionylglutaminyl-β-(pyrazolyl-3)-alanylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylprolylvaline Amide Acetate Hydrate (XVIII).—The protected eicosapeptide (110 mg) was dissolved in 90% TFA (1.5 ml) and the solution was kept at room temperature for 30 min. The TFA was evaporated, and the residue, dissolved in water (5 ml), was passed through a column (0.5 × 30 cm) of Amberlite IRA-400, which was washed with H_2O until the eluate was Cl-negative. Cl-positive eluates were pooled and lyophilized. This material dissolved in 0.25 *M* (pH 8.0) NH_4OAc (1 ml) was applied to a CMC column; (1 × 20 cm), which was eluted with an NH_4OAc gradient produced by mixing 100 ml of 0.25 *M* NH_4OAc (pH 8.0) with 100 ml of 0.25 *M* NH_4OAc (pH 10.5). Fractions (3 ml each) were collected at a flow rate of 1 ml/min. Absorbancy at 280 mμ located the desired material in tubes 34–130. These fractions were pooled, the bulk of the solvent was removed, and the residue was lyophilized to constant weight.

This material was reduced with thioglycolic acid:[23] 75 mg; $[\alpha]^{27}D$ −62.1° (*c* 1.02, 10% AcOH; R_f^I origin; R_f^{III} 0.5; single Cl-, Sakaguchi-, and ninhydrin-positive spot (See Figure 1 for disc electropherogram); amino acid ratios in acid hydrolysate: $Ser_{2.2}Tyr_{0.9}Met_{1.0}Glu_{1.0}Pyr(3)ala_{0.9}Phe_{1.0}Arg_{3.0}Gly_{2.0}Lys_{3.0}Pro_{2.0}Val_{2.2}$; amino acid ratios in AP-M digest: $Ser_{2.0}Tyr_{0.9}Met_{1.0}Gln_{1.1}Pyr(3)ala_{0.8}Phe_{0.9}Arg_{2.9}Trp_{0.9}Gly_{2.2}Lys_{3.1}Pro_{2.1}Val_{2.2}$ (84%).

Seryltyrosylserylmethionylglutaminylhistidylphenylalanylarginylphenylalanylglycyllysylprolylvalylglycyllysyllysylarginylarginylprolylvaline Amide Acetate Hydrate (XIX).—The dried tosylate salts derived from 246 mg of XXII and 305 mg of XXIII were dissolved in a mixture of DMF (12 ml) and pyridine (6 ml) and DCI (74 mg) was added. The reaction mixture was kept at room temperature for 4 days, and additional quantities of DCI (100 mg each) were added on the 2nd, 3rd, and 4th days. The bulk of the solvents was removed *in vacuo*, EtOAc (150 ml) was added to the residue and the suspension was kept in a

refrigerator for 2 hr. The precipitate was collected, washed with EtOAc, and dried. This material dissolved in 50% aqueous THF (50 ml) was passed through a column of Amberlite IRA-400 (2 × 14 cm), which was washed with the same solvent until the effluent was Cl-negative. Cl-positive eluates were pooled, the solution was evaporated to a small volume and lyophilized; 564 mg.

This material was dissolved in 90% TFA (15 ml) and the mixture was kept at room temperature for 40 min. The TFA was evaporated, the residue was lyophilized from H_2O (20 ml) and trifluoroacetate ions were exchanged for AcO^- in the usual manner.

The residue dissolved in 50 ml of 0.25 *M* NH_4OAc (pH 8.0) was applied to a CMC column (1.9 × 30 cm), which was developed with a gradient produced by mixing 1000 ml of 0.25 *M* (pH 8.0) NH_4OAc and 1000 ml of 0.25 *M* (pH 10.6) NH_4OAc. Fractions (10 ml each) were collected at a flow rate of 40 ml/hr. Fractions 85–140 were pooled, evaporated, and lyophilized to constant weight from H_2O; 210 mg. This material was rechromatographed in the same manner. Fractions containing the desired peptide, contaminated with sulfoxide, were pooled and desalted on a Bio-Rex 70 column (1.8 × 4.0 cm) using 150 ml of 5%, 350 ml of 10%, and 400 ml of 20% AcOH for elution. Absorbancy at 280 mμ located the desired product in the 20% AcOH eluates. These fractions were pooled, evaporated to a small volume, and lyophilized. The resulting material was reduced with thioglycolic acid:[28] 35 mg; $[\alpha]^{20}D$ −64.6° (*c* 1.40, 10% AcOH); R_f[11] 0.5, ninhydrin-, Cl-, and Pauly-positive spot; (see Figure 1 for disc electropherogram); amino acid ratios in AP-M digest: $Ser_{1.0}Tyr_{0.9}Met_{0.9}Glu_{1.2}His_{1.0}Phe_{1.0}Arg_{2.0}Gly_{6.0}Lys_{3.3}{}^{30}Pro_{2.1}Val_{2.0}$.

Results and Discussion

Table I summarizes the remarkable variety of amino acid substitutions in the N-terminal region of β-corticotropin fragments which are compatible with adrenocorticotropic and melanocyte expanding activity. The recorded activities must be regarded as approximations since they were determined in a number of laboratories under different conditions. Nonparallelism of the log dose–responses between standard and analog constitutes an additional source of error.

In order to evaluate the role of various amino acid residues in a biologically active peptide we have divided these residues into two classes. "Functional" or "active" residues are those whose replacement totally destroys biological activity; "binding sites" comprise residues whose substitution alters but does not totally destroy biological function.

Amino acid residues which are of critical functional significance for hormonal activity, if they exist, should be found within the sequence of β-corticotropin$_{1-20}$ amide (XVI)[9] which represents the smallest fully active fragment yet discovered.

It is difficult to correlate certain earlier observations, based on biological evaluation of chemically modified natural β-corticotropin, with more recent results obtained from structure–function studies with well defined synthetic fragments.

White[31] incubated β-corticotropin with leucine aminopeptidase and observed a rapid inactivation which appeared to parallel the liberation of serine[1] and tyrosine.[2] Selective N-acetylation of the N-terminal serine[32] or IO_4^- oxidation followed by reduction with BH_4^-, reactions which convert the N-terminal serine into a glyoxylyl group, resulted in almost complete inactivation.[33,34] These findings pointed to the N-terminal serine as an essential structural element for biologic function. However, glycine[1]-β-corticotropin, prepared from the glyoxylyl derivative by transamination, exhibited significant adrenocorticotropic activity.[35] This experiment eliminated the $HOCH_2$ group of the N-terminal serine as functionally essential, a result which is confirmed by the observation that synthetic Gly[1]-β-corticotropin$_{1-23}$ amide (XII)[36] is highly active. Complete elimination of the N-terminal serine (XV)[36] lowers but does not destroy adrenocorticotropic function. Replacement of the N-terminal serine by D-serine (IV),[37] D-alanine (V),[37] and β-alanine[38] affords analogs which possess an apparently higher biological potency than β-corticotropin$_{1-23}$ (III).[11]

Adrenocorticotropic activity is also retained when Tyr[2] is replaced by Phe (XIII)[36] and D-Ser[1]-D-Tyr[3]-β-corticotropin$_{1-23}$ (VI)[37] exhibits the full potency of β-corticotropin$_{1-23}$. Substitution of Ser[3] by Ala (XIV)[36] leads to a 50% decrease in adrenal ascorbic acid depleting activity.

The results of methionine modifications and replacements are particularly revealing since different alterations of this residue bring about apparently conflicting biological effects. Oxidation of the methionine S to SO destroys adrenocorticotropic potency of β-corticotropin[39] suggesting an intimate connection between the oxidation state of the methionine S and physiological function. However, substitution of methionine by Abut (XVII),[12,21,40] Nle (II),[41] or Leu[38] does not eliminate biological activity. Clearly the methionine S is not functionally essential despite the fact that its oxidation brings about inactivation. The available evidence points to the methionine residue as a hydrophobic binding site, but it is difficult to fit the high biological activity of D-Ser[1]-D-Tyr[2]-D-Ser[3]-D-Met[4]-β-corticotropin$_{1-23}$ (VII)[37] into this concept.

The rat adrenal ascorbic acid depletion assay showed XVIII to possess approximately 50 IU/mg. As concerns steroidogenic potency in the hypophysectomized or dexamethasone-blocked rat, the compound exhibited approximately 60% of the biological activity of corticotropin A_1 on a weight basis. Accurate evaluation of potency was difficult because of nonparallelism in the log dose response slopes between peptide and standard. The mean value of 3-hr plasma 11-hydroxycorticosteroid levels of 8 human subjects was raised from 22 μg/100 ml to 42 μg/100 ml when 0.75 mg of XVIII was administered i.v. over a 6-hr period. The urinary 17-keto steroid:creatinine ratio increased from 6.2 to 9.8 in the same period. At this dose level the compound failed to increase the blood levels of insulin, growth hor-

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mone, or glucose. No significant rise in 11-hydroxycorticosteroids or 17-keto steroids occurred when the same dose was given intramuscularly. The *in vitro* melanocyte expanding activity of XVIII was 8.4×10^7 MSH units/g.

The present observation, that replacement of histidine by β-(pyrazolyl-3)-alanine (XVIII) does not destroy the adrenocorticotropic and melanocyte expanding activities of XVI, clearly eliminates the acid–base properties of the imidazole ring as essential for these biological functions. Dedman, *et al.*,[17] suggested, on the basis of photolysis experiments, that histidine may not be important for the biological activity of ACTH. Our results confirm this earlier conclusion.

Substitution of arginine[8] by lysine or ornithine reduces drastically the adrenocorticotropic and melanocyte expanding properties of β-corticotropin fragments. Chung and Li[42] replaced arginine[8] in β-corticotropin$_{1-17}$ amide by lysine and found that this substitution decreased *in vitro* steroidogenic activity from approximately 44 to less than 1 U/mg. The *in vitro* melanocyte expanding potency was decreased from 2×10^8 U/g to 1×10^7 U/g.

The substitution by ornithine of Arg[8] in β-corticotropin$_{1-24}$ (IX) decreases *in vivo* adrenocorticotropic activity from 100 U/mg to 1 U/mg.[43] In this connection it is of interest to note that the arginine residues in positions 17 and 18 are not critical since they can be substituted by ornithine without loss in potency. Orn[17]-Orn[18]-β-corticotropin$_{1-24}$ exhibits the full adrenocortical potency of β-corticotropin$_{1-24}$.[44] The sequence Lys-Lys-Arg-Arg (positions 15 to 18) has long been regarded as a binding site.[45]

The replacement of tryptophan by phenylalanine in Gln[5]-β-corticotropin$_{1-20}$ amide compound (XIX) is accompanied by a marked diminution of *in vivo* adrenocorticotropic activity. The biological potency of the analog was 2 to 3% that of porcine β-corticotropin irrespective of whether activity was determined by adrenal ascorbic acid depletion or steroidogenesis. The *in vitro* melanocyte expanding activity of XIX was 10^7 MSH units/g.

Interpretation of this finding is not possible on the basis of available information, but poor binding to the receptor(s) may explain the low potency of XIX. The observation that XIX exhibits some biological activity argues against a direct functional involvement of the indole ring. The previously mentioned inactivation of β-corticotropin by *p*-dimethylaminobenzaldehyde[17] may also be the result of poor binding to the receptor(s) of the *p*-dimethylaminobenzylidene-β-corticotropin derivative which is presumably formed.

Irrespective of interpretation the available data point to the sequence Arg-Trp (positions 8 and 9) as a "sensitive" region of the hormone molecule.

Steric incompatibility to form a functioning hormone–receptor complex would explain the biological inertness of D-Glu[5]-D-His[6]-D-Phe[7]-D-Arg[8]-D-Trp[9]-β-corticotropin$_{1-24}$[37] (VIII), but the observation by Birnbaumer and Rodbell[46] that this compound antagonizes β-corticotropin competitively in rat fat-cell ghosts argues against this interpretation.

Evaluation of the effect of amino acid replacements on biological activity has not yet produced meaningful answers pertaining to specific amino acid residues which are connected critically with the biological activity of the β-corticotropin molecule. Moreover, these studies have contributed little to an understanding of the mode of action of this hormone. Many of the recorded substitutions have resulted in compounds which retain biological activity and in some instances (peptides II, IV, and V) adrenal ascorbic acid depleting activity was apparently enhanced. This apparent enhancement was attributed to increased resistance of the analogs to certain proteolytic enzymes, particularly aminopeptidases, with concomitant prolongation of action. When administered i.m. to man, the steroidogenic potency of peptide II was lower than that of porcine β-corticotropin and the synthetic peptide was not longer acting.[47]

A meaningful explanation of structure–function studies depends on the discovery of a more refined *in vitro* test system which will allow the differentiation between binding and function and which should eliminate the uncertainties inherent in the testing of these compounds in highly organized biological systems.[7] Exploration of the chemical nature of the ACTH receptors appears to offer a promising approach since ignorance pertaining to these structures retards progress in this field.

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