Chemical Modification of the Tryptophan Residue in Adrenocorticotropin†

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ABSTRACT: The single tryptophan residue in the pituitary hormone adrenocorticotropin was modified selectively by reaction with a variety of substituted *o*-nitrophenylsulfenyl chlorides. In addition to quantitative modification of the tryptophan residue, the reaction invariably resulted in partial oxidation of the methionine residue to the sulfoxide. The methionine sulfoxide derivative could be separated from the desired product by partition chromatography on Sephadex G-50 in the solvent system 1-butanol–pyridine–0.1% acetic acid (5:3:11). Thus, the 2,4-dinitrophenylsulfenyl, 2-nitro-4-carboxyphenylsulfenyl, and 2-nitro-4-carbamidophenylsulfenyl derivatives of adrenocorticotropin were prepared and characterized. Modifications in the isolation of adrenocorticotropin from ovine pituitaries are also described. The melanocyte stimulating activities of the native hormone and the analogues are discussed.

Selective chemical modification of the single tryptophan residue in ACTH[1] by reaction with *o*-nitrophenylsulfenyl chloride resulted in profound alteration of the biological properties of the hormone (Ramachandran, 1973). NPS-ACTH was found to be a potent inhibitor of ACTH-induced lipolysis in isolated rat adipocytes (Ramachandran and Lee, 1970a) as well as adenylate cyclase activity in rat fat cell ghosts (Ramachandran and Lee, 1970b). On the other hand, NPS-ACTH was three times as active as ACTH in stimulating adenylate cyclase activity in rabbit fat cell ghosts (Ramachandran and Lee, 1970b) and as a melanophore stimulating agent (Ramachandran, 1970). The steroidogenic potency of NPS-ACTH was found to be only 1.4% of ACTH in isolated adrenal cells and the analogue inhibited adenylate cyclase stimulation in rat adrenal cells (Moyle et al., 1973). It was apparent from these studies that the integrity of the tryptophan residue in ACTH is of great importance for the action of the hormone on rat adrenal cells and adipocytes. Furthermore, the tryptophan residue appears to be a sensitive site in the hormone which can be manipulated by selective chemical modification to reveal subtle differences in the structural requirements for productive interaction with the receptors of different target organs and species (Ramachandran, 1973, 1974). Therefore, we have introduced a variety of substituents into the 2 position of the indole ring of the tryptophan residue of ACTH by reaction with different nitrophenylsulfenyl chlorides. The preparation and characterization of DNPS-ACTH, NCPS-ACTH, and NCMPS-ACTH are described in this article. All the chemical modifications were performed on ACTH isolated from ovine pituitaries (Pickering et al., 1963). During this investigation, the isolation of ovine ACTH was modified in order to obtain better yields. These modifications are also described below.

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; NPS, 2-nitrophenylsulfenyl; DNPS, 2,4-dinitrophenylsulfenyl; NCPS, 2-nitro-4-carboxyphenylsulfenyl; NCMPS, 2-nitro-4-carbamidophenylsulfenyl; CM-cellulose, carboxymethylcellulose; BAW, 1-butanol–HOAc–H_2O (4:1:1, v/v).

Materials and Methods

NPS-Cl (Hubacher, 1943) was obtained from Eastman Organic Chemicals and recrystallized from chloroform (mp 71–73 °C). DNPS-Cl was obtained from J. T. Baker (mp 96–99 °C). NCPS-Cl was prepared as described by Havlik and Kharasch (1955) and recrystallized from 1,2-dichloroethane (dec 170 °C). Carboxymethylcellulose was purchased from Bio-Rad and Sephadex G-50 from Pharmacia Fine Chemicals, Inc. Acid protease was purchased from Miles Laboratories, Inc., and leucine aminopeptidase was from Worthington Biochemical Corp.

A Beckman Model DB-GT grating spectrophotometer was used for absorbance measurements at single wavelengths. Disc electrophoresis on 7% polyacrylamide gel was carried out at pH 4.5 (Davis, 1964). Continuous spectra were obtained with a Beckman Model DK-2A ratio recording spectrophotometer. Amino acid analyses were performed according to the method of Spackman et al. (1958) on a Beckman-Spinco Model 120 amino acid analyzer.

Melting points were determined on a Fisher-Johns block and were not corrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

Preparation of 2-Nitro-4-carbamidophenylsulfenyl Chloride (NCMPS-Cl). 2-Nitro-4-carboxy-*N*-benzylphenylsulfenamide (I). One gram of NCPS-Cl was dissolved in 20 ml of ethyl acetate with gentle heating. Two equivalents of benzylamine was added dropwise. The reaction was allowed to proceed in the dark at room temperature for 2 h with occasional shaking. The benzylamine-HCl was extracted with water and the ethyl acetate solution dried over anhydrous sodium sulfate. The sodium sulfate was removed by filtration and the resulting solution was evaporated to dryness in vacuo. The product was recrystallized from ether (mp 184–185 °C). Thin-layer chromatography on silica gel G in $CHCl_3$–CH_3OH (1:1) revealed one spot, R_f 0.60. Anal. Calcd for $C_{14}H_{12}N_2SO_4$: C, 55.25; H, 3.97; N, 9.21. Found: C, 55.07; H, 4.00; N, 9.11.

2-Nitro-4-carbamido-*N*-benzylphenylsulfenamide (II). Two equivalents of triethylamine was added to a solution of 0.6 g of I in 20 ml of ethyl acetate. The reaction flask was immersed in an ice bath, and isobutyl chloroformate (1.5 equiv) was added with stirring and allowed to react for 15

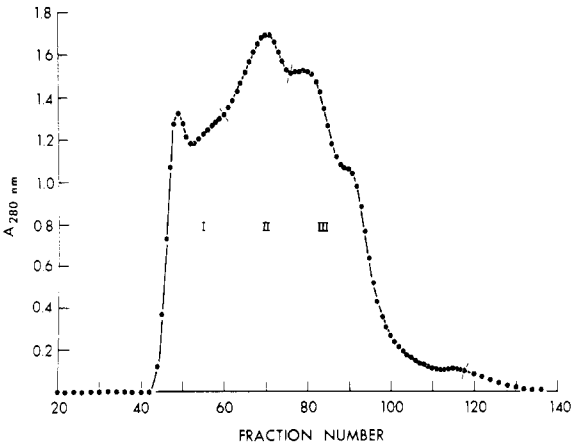


FIGURE 1: Gel filtration of ACTH fraction D′ on a Sephadex G-50 column (4 × 90 cm) in 1 N formic acid; flow rate, 60 ml/h; 10 ml/tube.

min. Ammonia was then bubbled through the solution for 15 min. The mixture was evaporated to dryness in vacuo and then dissolved in a minimum volume of ethyl acetate. The by-products were extracted with 5% sodium bicarbonate and the ethyl acetate was then washed with water to neutrality and dried over sodium sulfate. The sodium sulfate was removed by filtration and the resulting solution was evaporated to dryness in vacuo. The product was recrystallized from ether (mp 157–162 °C). Thin-layer chromatography in $CHCl_3$–CH_3OH (1:1) revealed a single spot, R_f 0.68. Anal. Calcd for $C_{14}H_{13}N_3SO_3$: C, 55.43; H, 4.32; N, 13.85. Found: C, 54.98; H, 4.13; N, 13.94.

2-Nitro-4-carbamidophenylsulfenyl Chloride (NCMPS-Cl). Two equivalents of HCl in ethyl acetate was added to a solution of 0.5 g of II in 20 ml of ethyl acetate and allowed to react for 15 min. The solution was evaporated to dryness in vacuo and the product was desiccated over NaOH. The sulfenyl chloride was recrystallized from 1,2-dichloroethane (dec 145 °C).

Preparation of the NCPS Derivative of N-Acetylglycyltryptophanamide. Glycyltryptophanamide-HCl (Fox Chemical Co.) (115 mg) was dissolved in 5 ml of anhydrous pyridine, cooled in ice, and allowed to react with 1 ml of acetic anhydride for 3 h. The solvent was removed in vacuo and the residue was dissolved in 10 ml of ethyl acetate and washed with 0.1 N HCl (2 × 10 ml). The aqueous phase was saturated with NaCl and extracted with ethyl acetate (2 × 10 ml). The combined extracts of ethyl acetate were washed with saturated NaCl solution and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo at room temperature and the residue was dissolved in 2.5 ml of 80% acetic acid. The product was found to be homogeneous by thin-layer chromatography in 1-butanol–HOAc–H_2O (4:1:1, v/v) (BAW), R_f 0.60; ninhydrin negative, Ehrlich positive. Thin-layer chromatography of the starting material glycyltryptophanamide in BAW revealed a single ninhydrin and Ehrlich positive spot, R_f 0.42. The yield of *N*-acetylglycyltryptophanamide was estimated by the absorbance at 280 nm to be 67 mg.

To the solution of *N*-acetylglycyltryptophanamide in 80% acetic acid, 50 mg of NCPS-Cl was added and the reaction was allowed to proceed for 20 h at room temperature. The sulfenylated peptide was precipitated by the addition of 30 ml of ice cold water. The precipitate was filtered and washed with water and then with ether and finally dried in a desiccator over P_2O_5. The orange-yellow product was triturated with hot ethyl acetate, allowed to cool to room temperature, filtered, and washed with ethyl acetate to yield 56 mg of the NCPS derivative of *N*-acetylglycyltryptophanamide (dec 176–179 °C). Thin-layer chromatography of the product revealed a single, yellow spot, ninhydrin and Ehrlich negative in both BAW (R_f 0.62) and $CHCl_3$–CH_3OH (1:1, v/v), R_f 0.41.

Preparation of NCMPS Derivative of N-Acetyltryptophanamide. To a solution of 50 mg of *N*-acetyltryptophanamide (mp 192–194 °C) in 2 ml of glacial acetic acid, 50 mg of NCMPS-Cl was added at room temperature and allowed to react in the dark overnight. The product was precipitated by the addition of 30 ml of water, collected, and dried in vacuo over P_2O_5. Recrystallization was accomplished from ethyl acetate–petroleum ether (dec 188 °C). Thin-layer chromatography in BAW revealed a single spot, R_f 0.78.

Isolation of Ovine ACTH. A saturated NaCl precipitate (fraction D) was prepared from an acid–acetone extract of frozen whole ovine pituitary glands as previously described (Li, 1952) except that rapid dialysis was effected at pH 3.0 with a Bio-Rad hollow fiber dialyzer. From 500 g of pituitaries approximately 2 g, designated fraction D′, was obtained.

Separation of ACTH from an ACTH-binding protein (Nakamura and Tanaka, 1969) was accomplished by dissolving 1 g of fraction D′ in 1 N HCOOH and chromatographing at room temperature on a Sephadex G-50 column (4 × 90 cm) in 1 N HCOOH (Figure 1). The ACTH activity was found in fraction III, which was further fractionated on CM-cellulose as described by Birk and Li (1964). Rechromatography on CM-cellulose yielded 40 mg of highly purified ACTH.

Sulfenylation of Ovine ACTH. Forty milligrams of ACTH was dissolved in 0.2 ml of water plus 1.8 ml of glacial acetic acid. Fifty milligrams of the sulfenyl chloride in 1 ml of glacial acetic acid was added and the solution was kept in the dark with occasional shaking at room temperature. After 4 h the solution was diluted with 30 ml of water and extracted with ethyl acetate. After the organic phase was extracted with 0.1% acetic acid, the combined aqueous phase was lyophilized and yielded 50 mg. The derivative was purified by chromatography on a CM-cellulose column (1 × 25 cm) using an ammonium acetate gradient as described for ACTH (Pickering et al., 1963).

Partition Chromatography on Sephadex G-50. Sephadex G-50 fine was sieved on a U.S. Standard screen no. 325, and the material that passed through was used. Columns were initially packed in water. The column was then equilibrated with the lower phase of the solvent system 1-butanol–pyridine–0.1% aqueous acetic acid (5:3:11) previously employed in countercurrent distribution of ACTH (Dixon et al., 1961), followed by equilibration with the upper phase as described previously (Yamashiro, 1964). The peptide was dissolved in the upper phase and chromatographed by elution with the upper phase. Peptide material was detected by the Folin–Lowry procedure (Lowry et al., 1951) in the case of ACTH, whereas the ACTH analogues could be conveniently followed spectrophotometrically at 360 nm after dilution with 50% ethanol to ensure a one-phase system. For isolation, appropriate fractions were mixed with 2 vol of water, evaporated in vacuo to a small volume, and lyophilized.

Enzymatic Digestion. The peptide (0.5 mg) was incubated with 50 μg of acid protease in 0.2 ml of 0.01 N HCl at 37 °C for 20 h. After lyophilization, the sample was incubated

with 1.6 U of leucine aminopeptidase in 0.2 ml of 0.05 M Tris-HCl buffer (pH 8.5), containing 0.01 M $MgCl_2$ for 24 h at 37 °C. The sample was then analyzed on the amino acid analyzer.

Peptide Maps. The peptide (1 mg) was incubated with diphenylcarbamyl chloride treated trypsin (20 μg) in 0.2 ml of 0.2 M ammonium acetate (pH 8.0) at 37 °C for 8 h. After lyophilization the digest was subjected to descending chromatography on Whatman 3 mm paper in the upper phase of freshly prepared 1-butanol–acetic acid–water (4:1:5, v/v) for 20 h at room temperature. High voltage electrophoresis was performed in the second dimension at pH 2.1 in the buffer composed of 28% formic acid–acetic acid–water (218:63:719, v/v) for 50 min (35 V/cm). The peptide map was examined visually for yellow spots and then developed with ninhydrin.

Biological Assays. The steroidogenic potency of ACTH fractions was estimated by the in vivo procedure of Vernikos-Danellis et al. (1966) using hypophysectomized rats. Melanocyte-stimulating activity was measured in vitro by the frog skin method (Shizume et al., 1954; Ramachandran, 1970).

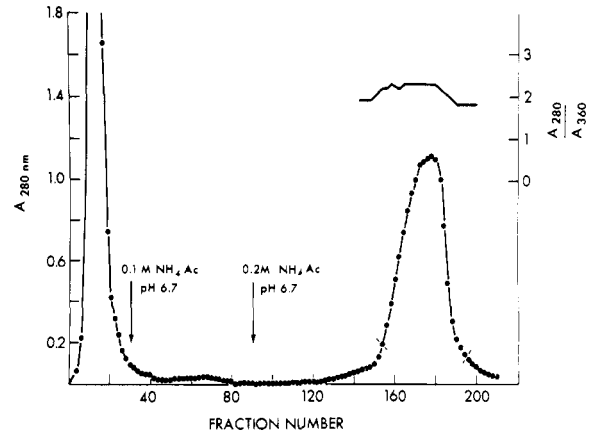


FIGURE 2: CM-cellulose chromatography of DNPS-ACTH. The peptide was applied on a column (1 × 25 cm) equilibrated with 0.01 M NH_4OAc (pH 4.5). After four hold-up volumes were collected with the same buffer, a gradient was started by introducing 0.1 M NH_4OAc (pH 6.7), through a 125-ml mixing chamber containing the starting buffer. Later, the gradient was increased by substitution of 0.2 M NH_4OAc (pH 6.7) as the solution flowing into the mixing chamber; flow rate, 60 ml/h; 2 ml/tube.

Results

Isolation of ACTH. In the improved procedures for the isolation of ACTH (Pickering et al., 1963; Birk and Li, 1964), the precipitate obtained by saturation with NaCl was dialyzed against running tap water at pH 9. At the end of the dialysis, the precipitate in the dialysis bag was discarded, and the supernatant was lyophilized and subjected to CM-cellulose chromatography. We traced our inability to obtain previously reported yields of ACTH to losses at the dialysis stage; most of the biological activity lost at this stage was found in the precipitate which was routinely discarded. The dialysis procedure was modified by using the hollow fiber device which retains small molecules. By performing the dialysis at pH 3 in the hollow fiber device, possible deamidation of ACTH (known to occur at slightly alkaline pH) was also avoided.

When the product of dialysis was chromatographed on CM-cellulose, the ACTH fraction was found to be heterogeneous. This was found to be caused by an interaction between ACTH and the unadsorbed material from the CM-cellulose chromatography. In order to dissociate any ACTH–protein complex, the dialysis product was subjected to gel filtration on Sephadex G-50 in 1 N formic acid (Figure 1). Fraction III contained the ACTH activity and chromatography of this fraction on CM-cellulose (2×) yielded a homogeneous preparation of ACTH. The yield of ACTH was 80 mg/kg pituitaries. The product was found to be homogeneous by electrophoresis on Whatman No. 1 paper at pH 3.7 (pyridine–HOAc–H_2O, 4:40:1156, v/v; 12 V/cm) for 5 h and by disc electrophoresis on polyacrylamide gel at pH 4.5. Amino acid analysis of an acid-hydrolyzed sample gave values in agreement with the established sequence of ovine ACTH (Table III). The steroidogenic activity was assayed by the in vivo procedure of Vernikos-Danellis et al. (1966). The potency of the hormone isolated by this modified method was 130% that of previously obtained ACTH with 95% confidence limits of 86–205% and an index of precision of 0.13.

Modification of the Tryptophan Residue. The modification of the indole moiety of the tryptophan residue in ACTH was accomplished by reaction with the appropriate sulfenyl chloride in 90% acetic acid. After extracting the by-products with ethyl acetate, the peptide derivative was chromatographed on CM-cellulose. DNPS-ACTH emerged as a single peak with a fairly constant 280/360 absorbance ratio, indicating the absence of unreacted hormone (Figure 2). Electrophoresis on Whatman No. 1 paper in 5% acetic acid (pH 2.6, 12 V/cm) for 4.5 h revealed single yellow, ninhydrin positive, Ehrlich negative spots for the ACTH analogues.

In order to assess the completeness of the reaction as well as selectivity, we employed enzymatic digestion of the peptide derivatives with an acid protease followed by leucine aminopeptidase. Although this procedure does not liberate all the amino acids in the ACTH derivatives, it causes the release of all the amino acids in the N-terminal decapeptide of the molecule which are considered crucial for the biological activities (Ramachandran, 1973). When DNPS-ACTH isolated after CM-cellulose chromatography was subjected to the enzymatic digestion procedure, no tryptophan residue was found but the yield of methionine was low and a small amount of methionine sulfoxide was present. Since rechromatography of DNPS-ACTH on CM-cellulose failed to resolve the methionine sulfoxide component, we resorted to partition chromatography in 1-butanol–pyridine–0.1% acetic acid (5:3:11, v/v) as described by Yamashiro (1964) and Yamashiro and Li (1973). This procedure successfully separated DNPS-ACTH into two major components (Figure 3). Enzymatic digestion followed by amino acid analysis revealed that the major fast-moving component (peak I) contained only methionine whereas the slower component (peak II) contained both methionine and methionine sulfoxide.

Similarly, CM-cellulose chromatography of NCMPS-ACTH yielded a main fraction with a constant 280/360 absorbance ratio (Figure 4). However, this material contained a small amount of methionine sulfoxide (Table I). Partition chromatography of this product yielded two well-separated fractions (Figure 5). The fast-moving fraction (peak I) had only methionine, whereas the slower component (peak II) contained considerable amounts of methionine sulfoxide (Table I). The yields of the sulfenylated ACTH analogues ranged from 50 to 60%.

Partition chromatography was useful not only in resolving the methionine derivative from the sulfoxide derivative,

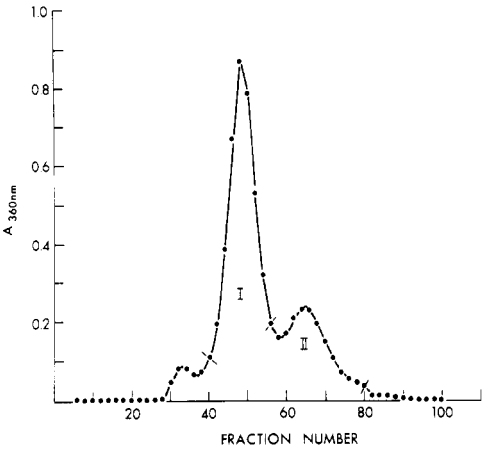


FIGURE 3: Partition chromatography of DNPS-ACTH on Sephadex G-50 (2 × 17 cm); flow rate, 11 ml/h; 0.6 ml/tube.

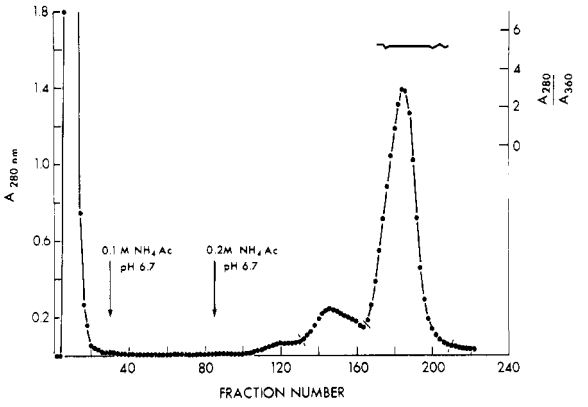


FIGURE 4: CM-cellulose chromatography of NCMPS-ACTH. Conditions are the same as those described in Figure 2.

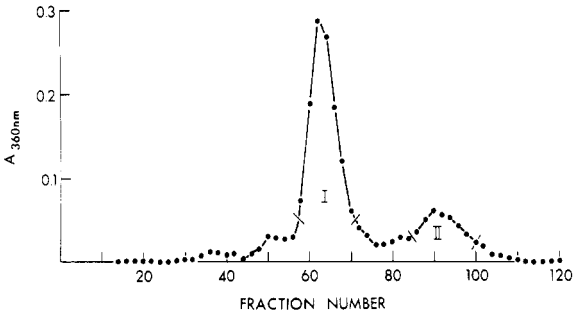


FIGURE 5: Partition chromatography of NCMPS-ACTH on Sephadex G-50 (2 × 37 cm); flow rate, 10 ml/h; 1 ml/tube.

but also in separating the unmodified hormone from the products of reaction with the different sulfenyl chlorides. The separation of DNPS-ACTH from ACTH is illustrated in Figure 6 and the R_f values of all the derivatives are listed in Table II.

The peptide derivatives isolated after partition chromatography were further characterized by amino acid analysis, absorption spectra, and gel electrophoresis. The amino acid composition of the acid hydrolysate and enzymatic digest for each of the derivatives is given in Table III together with the results obtained for ACTH. Tryptophan was absent in the hydrolysates of all the peptides except the enzymic digest of ACTH. Sulfenylated tryptophan is destroyed during acid hydrolysis (Scoffone et al., 1968). The extent of enzyme digestion of ACTH as well as the acid and enzyme

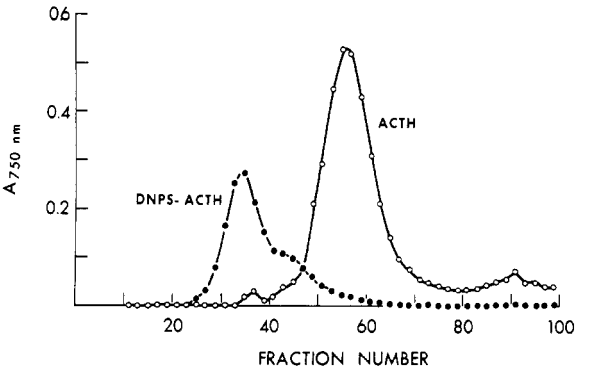


FIGURE 6: Partition chromatography of ACTH and DNPS-ACTH on Sephadex G-50 (1 × 19 cm); flow rate, 4 ml/h; 0.3 ml/tube.

Table I: Amino Acid Composition of Enzymatic Digests of NCMPS-ACTH Fractions.[a]

Amino Acid	Before Partition Chromatog.	After Partition Chromatog.	
		Peak I, Fig. 5	Peak II, Fig. 5
Trp	0.00	0.00	0.00
Lys	1.86	2.20	2.33
His	0.78	1.16	0.84
Arg	3.10	2.94	2.46
Asp	1.39	0.73	1.58
Ser + Gln + Asn	4.09	4.61	4.72
Glu	4.00	4.00	4.00
Pro	3.22	2.94	3.10
Gly	1.51	1.17	1.47
Ala	2.85	3.27	3.27
Val	2.15	2.55	2.96
Met	0.76	1.06	0.34
Met-SO	0.18	0.00	0.28
Leu	1.16	1.43	1.38
Tyr	1.87	1.85	2.03
Phe	2.79	2.86	3.29

[a] The peptide fractions were treated with acid protease and leucine aminopeptidase in sequence as described under Materials and Methods.

Table II: Partition Chromatography of ACTH and Derivatives.[a]

Peptide	R_f Value[b]
ACTH	0.34
NPS-ACTH	0.48
NPS-ACTH (Met-SO)	0.36
DNPS-ACTH	0.48
DNPS-ACTH (Met-SO)	0.36
NCPS-ACTH	0.33
NCPS-ACTH (Met-SO)	0.24
NCMPS-ACTH	0.40
NCMPS-ACTH (Met-SO)	0.27

[a] Partition chromatography on Sephadex G-50 was carried out as described under Materials and Methods. [b] R_f is defined as V_h/V_e where V_h is the hold-up volume and V_e is the elution volume of the peak.

digestion of the derivatives relative to acid hydrolysis of ACTH is also given in Table III. ACTH is almost completely digested by the enzymic procedure but the derivatives are hydrolyzed to the extent of 82–86%. This is reflected in the lower amounts of lysine, glycine, and valine found in the enzyme digests of the derivatives. The degree of acid

Table III: Amino Acid Composition of Acid and Enzymatic Hydrolysates of ACTH and Derivatives.

Amino Acid	ACTH		NPS-ACTH		DNPS-ACTH		NCPS-ACTH		NCMPS-ACTH	
	Acid[a]	Enzyme[b]	Acid	Enzyme	Acid	Enzyme	Acid	Enzyme	Acid	Enzyme
Trp	0.00 (1)[c]	0.86	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lys	4.26 (4)	3.51	3.87	2.28	3.72	2.11	3.61	2.30	3.54	2.20
His	1.04 (1)	1.05	0.89	0.84	0.76	0.94	0.83	1.03	0.89	1.16
Arg	3.00 (3)	2.69	3.00	3.05	3.00	3.01	3.00	3.26	3.00	2.94
Asp	2.04 (2)	1.56	2.01	0.94	2.17	1.63	2.12	1.35	2.21	0.73
Ser + Gln + Asn	2.70 (3)	3.86	2.32	4.01	2.57	4.22	2.61	4.14	2.64	4.61
Glu	5.00 (5)	4.00	5.00	4.00	5.00	4.00	5.00	4.00	5.00	4.00
Pro	4.78 (4)	3.53	4.12	4.49	3.28	3.09	3.61	3.42	3.27	2.94
Gly	3.34 (3)	2.72	3.10	1.18	2.60	1.95	2.69	1.66	2.58	1.17
Ala	3.07 (3)	3.01	3.00	3.42	3.23	3.01	3.17	2.94	3.16	3.27
Val	3.12 (3)	3.01	3.03	2.79	3.37	2.30	3.49	2.09	3.30	2.55
Met	1.03 (1)	0.87	1.13	0.89	0.94	0.90	0.17	1.21	0.19	1.06
Met-SO	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.26	0.00
Leu	1.14 (1)	1.15	1.02	1.09	1.27	0.89	1.28	1.22	1.26	1.43
Tyr	2.01 (2)	1.79	1.84	1.81	1.94	1.97	1.86	2.38	1.89	1.85
Phe	2.99 (3)	3.04	2.77	2.88	3.32	2.95	3.07	2.81	3.29	2.86
% digestion[d]	100	92.7	93.9	85.2	94.1	83.4	92.9	85.6	92.3	82.9

[a] The peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h. [b] The peptides were treated with acid protease and leucine aminopeptidase in sequence as described under Materials and Methods. [c] The theoretical values are shown in parentheses. [d] The percent digestion was calculated relative to acid hydrolysis of ACTH.

Table IV: Absorption Maxima and Molar Absorptivities of Tryptophan Peptides Modified by Reaction with Sulfenyl Chlorides.[a]

Peptide	λ_{max} (nm)	ϵ_{max} ($\times 10^{-3}$)
Ac-Gly-(NCPS)-Trp-NH_2	282	20.5
	360	4.22
Ac-(NCMPS)-Trp-NH_2	279	17.47
	360	4.48
NPS-ACTH	280	16.87
	361	3.88
DNPS-ACTH	275	18.69
NCPS-ACTH	278	19.45
	363	3.68
NCMPS-ACTH	276	18.37
	360	3.36

[a] All the spectra were recorded in 0.001 N HCl.

Table V: Electrophoretic Mobilities of ACTH and Derivatives on Polyacrylamide Gel.[a]

Peptide	Mobility Rel to ACTH
ACTH	1.00
NPS-ACTH	0.89
DNPS-ACTH	0.87
NCPS-ACTH	0.76
NCMPS-ACTH	0.93

[a] The peptides (100 μg each) were subjected to electrophoresis at pH 4.5 and the bands located by staining with Amido-Schwarz.

Table VI: Melanocyte-Stimulating Activities of ACTH Derivatives.

Peptide	Potency[a]
ACTH	100
NPS-ACTH	326 (212–488)
NCMPS-ACTH	206 (135–312)
NCPS-ACTH	121 (75–216)
DNPS-ACTH	66 (35–113)

[a] The activity of ACTH is taken as 100 and the potencies of the derivatives are expressed as percent activity relative to ACTH. The figures in parentheses represent the 95% confidence limits.

hydrolysis of the derivatives was found to be 92–94% that of ACTH and may reflect partial destruction of some amino acids during acid hydrolysis of the derivatives. Thus, methionine appears to be partially destroyed and partially converted to the sulfoxide derivative during acid hydrolysis of NCPS-ACTH and NCMPS-ACTH. However, the enzyme digests clearly show that the integrity of the methionine residue is fully preserved in all the derivatives isolated after partition chromatography. No trace of methionine sulfoxide or sulfone was found in the enzyme digests of ACTH or the purified derivatives.

Peptide maps of tryptic digests of DNPS-ACTH and NCPS-ACTH were prepared and compared with that of ACTH. The fingerprints were found to be identical except for the absence of a ninhydrin positive, Ehrlich positive spot in the maps of the derivatives and the appearance of a single new, yellow ninhydrin positive, Ehrlich negative spot in the maps of the derivatives.

Further evidence of substitution at a single site in the ACTH derivatives was obtained from the absorption spectra. The absorption maxima and molar absorptivities of the ACTH derivatives as well as some model compounds in 0.001 N HCl are given in Table IV. All the derivatives except DNPS-ACTH exhibit a maximum around 360 nm. The molar extinction coefficients at this absorption maximum are in good agreement with the molar extinction coefficients of model compounds modified only at the tryptophan residue (Table IV and Scoffone et al., 1968). The sulfenylated derivatives were also found to be homogeneous by polyacrylamide gel electrophoresis at pH 4.5 (Table V). As expected, NCPS-ACTH was found to be most retarded.

The melanocyte-stimulating activities of the derivatives are compared with those of the native hormone in Table VI. NPS-ACTH is seen to be the most potent derivative. NCMPS-ACTH is more active than NCPS-ACTH and both are more active than ACTH. Only DNPS-ACTH was found to be less potent than the native hormone.

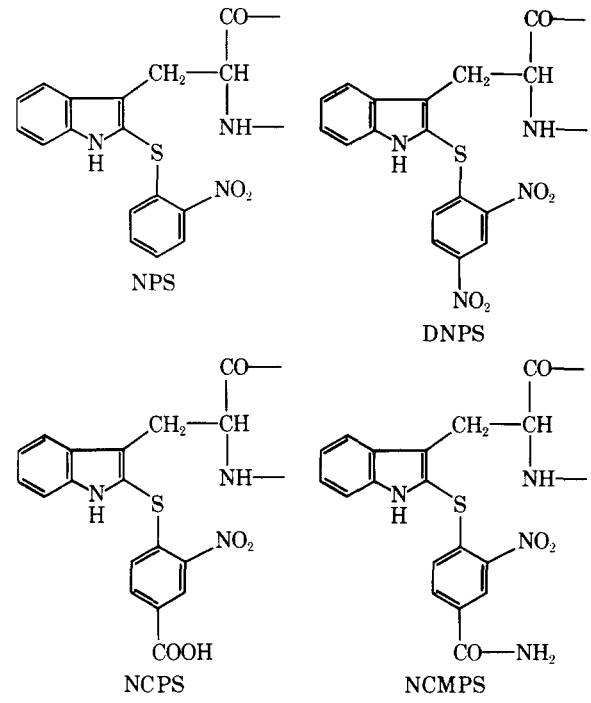
Discussion

o-Nitrophenylsulfenyl chloride has been widely used for the selective modification of tryptophan residues in a number of biologically active polypeptides and proteins (Scoffone et al., 1968; Brovetto-Cruz and Li, 1969; Ramachandran and Lee, 1970a; Ramachandran, 1970; Kawauchi et al., 1973). In the absence of sulfhydryl groups the reaction at acidic pH is highly specific for the indole group of the tryptophan residue. Scoffone et al. (1968) reported quantitative recovery of all the amino acids except tryptophan, cysteine, and methionine after reaction of a standard amino acid mixture with NPS-Cl in acetic acid for 15 h. Even a short reaction time (20 min) caused partial conversion of methionine to the sulfoxide. Previous studies have shown that the biological activities of ACTH are affected significantly by the conversion of the single methionine residue to the sulfoxide and that the biological activity is restored following reduction of the sulfoxide (Dedman et al., 1961). In view of this, it was important to ascertain the integrity of the methionine residue after reaction with the sulfenyl chlorides.

Since acid hydrolysis results in the conversion of methionine sulfoxide to methionine (Ray and Koshland, 1960), we resorted to enzymatic hydrolysis of the derivatives by a combination of acid protease and leucine aminopeptidase. Initial cleavage of the peptides with acid protease rather than trypsin or chymotrypsin was chosen owing to the poor solubility of the ACTH derivatives at neutral pH. It was found that reaction of ACTH with a sulfenyl chloride was always accompanied by partial oxidation of the methionine residue. Recrystallization of the sulfenyl chloride prior to use tended to decrease oxidation of methionine to the sulfoxide but did not completely eliminate this side reaction.

Separation of the methionine sulfoxide derivative of the modified ACTH from the desired product, therefore, became essential. CM-cellulose chromatography failed to separate the sulfoxide component but partition chromatography in 1-butanol–pyridine–acetic acid effected the separation of the desired product from the sulfoxide derivative in every instance. Amino acid analysis of enzyme digests of each of the sulfenylated ACTH derivatives showed that only methionine was present in the peptides after purification by partition chromatography (Tables I and III). The retarded minor component in partition chromatography in each case (peak II, Figure 3, and peak II, Figure 5) was found to contain methionine sulfoxide as well as methionine as shown by the amino acid composition of the enzyme digests (Table I and unpublished observations). The presence of methionine in peak II, Figure 3, is understandable owing to the incomplete resolution of peaks I and II. The presence of methionine in addition to methionine sulfoxide in peak II, Figure 5 [NCMPS-ACTH (Met-SO)], may reflect the presence of some NCPS-ACTH in this peak. The NCPS-ACTH could have arisen from deamidation of NCMPS-ACTH. CM-cellulose chromatography of NCMPS-ACTH (Figure 4) shows the presence of a minor component which emerged ahead of the major peak and which was not fully resolved from the major peak. The R_f values of NCPS-ACTH and NCMPS-ACTH (Met-SO) (Table II) indicate that these two cannot be resolved by partition chromatography. The lower amounts of glycine, lysine, and valine found in the enzyme digests of all the sulfenylated ACTH derivatives indicate that the sequence Trp-Gly-Lys-Pro-Val-Gly-Lys may be digested slowly or incompletely after modification of the tryptophan residue.

Scheme I: Structures of the Substituents Introduced at the 2 Position of the Indole Ring of ACTH by Reaction with Different Sulfenyl Chlorides.



The side reaction is of concern in the modification of polypeptides like ACTH with a random conformation in which the methionine residues are exposed. In globular proteins where the methionine side chains are usually buried in the hydrophobic interior of the molecule, this side reaction is not likely to be significant.

The structures of the different substituents introduced at the 2 position of the indole ring of ACTH are depicted in Scheme I. Whereas NPS-ACTH is 326% as active as ACTH as a melanocyte-stimulating agent, DNPS-ACTH is 66% as active. The introduction of the second nitro group into this long polypeptide chain caused a drastic alteration in this biological activity. NCPS-ACTH containing a carboxyl group in the phenyl ring was also much less active than NPS-ACTH, although slightly more active than ACTH itself (121%). The NCMPS derivative was prepared in order to see if the negative charge contributed by the carboxyl group in NCPS-ACTH was responsible for the significant decrease in activity compared to NPS-ACTH. NCMPS-Cl was synthesized from NCPS-Cl by treating first with benzylamine, then converting the carboxyl group to an amide by the mixed anhydride procedure, and finally removing the benzylamine group by HCl in ethyl acetate. Indeed, the melanocyte-stimulating activity of NCMPS-ACTH was higher than that of NCPS-ACTH (206% as active as ACTH) but still not as high as that of NPS-ACTH. These results bear out the expectations that the tryptophan residue of ACTH is a highly sensitive region in the molecule. The introduction of different substituents at this site can serve to probe the nature of the receptors with which the hormone interacts in the various target cells. The effects of these closely related derivatives of ACTH on isolated rat adrenal cells and isolated fat cells of the rat and rabbit are under study and will be reported in detail elsewhere.

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References

Birk, Y., and Li, C. H. (1964), *Biochim. Biophys. Acta 82,* 430.

Brovetto-Cruz, J., and Li, C. H. (1969), *Biochemistry 8,* 4659.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Dedman, M. L., Farmer, T. H., and Morris, C. J. O. R. (1961), *Biochem. J. 78,* 348.

Dixon, J. S., Lo, T. B., and Li, C. H. (1961), *Arch. Biochem. Biophys. 92,* 296.

Havlik, A. J., and Kharasch, N. (1955), *J. Am. Chem. Soc. 77,* 1150.

Hubacher, M. H. (1943), Organic Syntheses, Collect. Vol. II, New York, N.Y., Wiley, p 455.

Kawauchi, H., Bewley, T. A., and Li, C. H. (1973), *Biochemistry 12,* 2124.

Li, C. H. (1952), *Acta Endocrinol. 10,* 255.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Moyle, W. R., Kong, Y. C., and Ramachandran, J. (1973), *J. Biol. Chem. 248,* 2409.

Nakamura, M., and Tanaka, A. (1969), *Endocrinol. Jpn. Suppl. 1,* 53.

Pickering, B. T., Andersen, R. N., Lohmar, P., Birk, Y., and Li, C. H. (1963), *Biochim. Biophys. Acta 74,* 763.

Ramachandran, J. (1970), *Biochem. Biophys. Res. Commun. 41,* 353.

Ramachandran, J. (1973), *Horm. Proteins Pept. 3,* 1–28.

Ramachandran, J. (1974), *Isr. J. Chem. 12,* 675.

Ramachandran, J., and Lee, V. (1970a), *Biochem. Biophys. Res. Commun. 38,* 507.

Ramachandran, J., and Lee, V. (1970b), *Biochem. Biophys. Res. Commun. 41,* 358.

Ray, W. J., Jr., and Koshland, D. E., Jr. (1960), *Brookhaven Symp. Biol. 13,* 135.

Scoffone, E., Fontana, A., and Rocchi, R. (1968), *Biochemistry 7,* 971.

Shizume, K., Lerner, A. B., and Fitzpatrick, T. B. (1954), *Endocrinology 54,* 553.

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

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

Yamashiro, D. (1964), *Nature (London) 201,* 76.

Yamashiro, D., and Li, C. H. (1973), *J. Am. Chem. Soc. 95,* 1310.