Group	1	2	3	4
Administered material	0	medium with fraction HH_{9+1}	medium after incubation of hypophyses without HH_{9+1}	medium after incubation of hypophyses with HH_{9+1}
Number of tests	16	14	14	15
Concentration of fraction $HH_9{}^+{}_1$ in the medium	—	1 cm³/3.3 cm³	0	1 cm³/3.3 cm³
Total dose of tested material in cm³	0	2	2	2
Mean weight of adenohypophyses incubated in 1 cm³ medium in mg	—	—	6.6	6.6
% of the dose of radio-iodine found in the thyroid gland. Means $\pm \sigma_m$	0.9 ± 0.10	1.3 ± 0.18	2.2 ± 0.33	5.1 ± 0.87 [a]

[a] Comparison of groups 3 and 4 by Fisher's t-test: $p < 0.01$

Krebs-Ringer-phosphate and 3 cm³ fraction HH_{9+1} (second control group); (3) 14 hypophysectomized rats received four doses of 0.5 cm³ of medium A (after incubation of the hypophyses without fraction HH_{9+1}); (4) 15 hypophysectomized rats received four doses of 0.5 cm³ of medium B (after incubation of the hypophyses with fraction HH_{9+1}).

1 h after the last injection 4 μC of carrier-free $Na^{131}I$ was administered to each rat subcutaneously. 24 h after the administration of radio-iodine, the rats were killed by ether anaesthesia, the thyroid glands were removed and their radioactivity was measured by Geiger-Müller counter. The activity of each thyroid was expressed as a percentage of the radio-iodine dose administered, a model of the rat thyroid containing 4 μC $Na^{131}I$ being used as the indicator of 100% accumulation.

The results are shown in the Table. The radio-iodine uptake in rats of group 2 was not statistically different from group 1, indicating that no TSH was present in fraction HH_{9+1}. After the administration of medium A (after incubation of the adenohypophyses without fraction HH_{9+1}), the radio-iodine uptake doubled, indicating some release of TSH into the medium. After the administration of medium B, a substantial elevation in thyroid radio-iodine uptake was registered, idincating an at least two-fold increase in TSH release into the medium in the presence of fraction HH_{9+1}. It appears, therefore, that fraction HH_{9+1} stimulated the release of TSH from rat adenohypophysial tissue *in vitro*. This is in agreement with our previous results on the effect of the original non-protein fraction HH_9 on TSH release from adenohypophysial autografts *in vivo*[3]. Further work is needed to prove the specificity of this reaction and to test whether only release or also the production of TSH is influenced.

Zusammenfassung. Die elektrophoretisch isolierte Fraktion des hypothalamischen Extraktes erhöht *in vitro* die Sekretion des thyreotropen Hormons aus der Adenohypophyse der Ratte in der Krebs-Phosphat-Ringerlösung mit 300 mg% Glukose. Die Fraktion selbst besitzt keine TSH-Wirkung.

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Influence of the Structure of the *N*-terminal Extremity of α-MSH on the Melanophore Stimulating Activity of this Hormone

We have formerly reported[1,2] the total synthesis of α-MSH[3,4]. When compared with natural hog α-MSH[5], our synthetic material has shown the same melanophore stimulating activity by both the *in vivo*[6] and the *in vitro*[7] assays[8,9], the same kinetics of degradation by chymotrypsin and by trypsin, and the same chromatographic and electrophoretic behaviour under a variety of conditions, thus definitely proving its complete identity with the natural product. We have also found that our synthetic product loses its biological activity under the action of hydrogen peroxyde and regains it almost completely by subsidiary treatment with cysteine or thioglycollic acid[10].

This successful synthesis of α-MSH opened the way for obtaining synthetic analogues of this hormone. By following a scheme of synthesis similar to that one we used for α-MSH, we have prepared the three structural analogues (A, B, and C) mentioned in the Table, in order to investigate the influence of the structure of the *N*-terminal extremity on the biological properties[11].

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[4] J. I. Harris and A. B. Lerner, Nature *179*, 1346 (1957). – J. I. Harris, Biochem. J. *71*, 451 (1959).

[5] We are indebted to Dr. R. Guillemin and Dr. A. V. Schally (Baylor University, Houston) for a generous gift of pure natural α-MSH.

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

[8] The assays were kindly performed by Dr. Flückiger in our Pharmacological Department.

[9] We thank Dr. Guillemin for having kindly duplicated our assays in his own laboratory.

[10] H. B. F. Dixon, Biochim. biophys. Acta *19*, 392 (1956).

[11] A preliminary account of this work was given at the 17th International Congress of Pure and Applied Chemistry (August 20 to September 6, 1959, Munich), Abstract B 230, and at the 2nd European Peptide Symposium (September 6 to 9, 1959, Munich), Angew. Chemie *72*, 47 (1960).

Using the dicyclohexylcarbodiimide method[12], we condensed CBO-Ser-Tyr-Ser-Met-Glu(OBz)-OH[13,14] with H-His-Phe-Arg-Try-Gly-(ε-CBO)Lys-Pro-Val-NH_2[15] in acetonitrile/dimethylformamide 2:1 to CBO-Ser-Tyr-Ser-Met-Glu(OBz)-His-Phe-Arg-Try-Gly-(ε-CBO)Lys-Pro-Val-NH_2 (85% yield. M.p. 160°. Analysis calculated for $C_{98}H_{126}O_{22}N_{21}SCl$: C 58.3; H 6.3; O 17.5; N 14.6; S 1.6. Found: C 59.2; H 6.5; O 17.2; N 14.3; S 1.7. K = 15 in the system sec-butanol/trichloracetic acid/water 200:1:200. UV spectrum: λ_{max} = 282 mμ ($\log\varepsilon$ = 3.83), 292 mμ ($\log\varepsilon$ = 3.70). Amino acid composition: $Ser_{2.2}$ $Tyr_{1.0}$ $Met_{1.0}$ $Glu_{1.0}$ $His_{0.9}$ $Phe_{1.0}$ $Arg_{0.9}$ $Gly_{1.1}$ $Lys_{1.2}$ $Val_{1.1}$).

Scission of the protecting groups was accomplished by passing a current of dry hydrogen bromide into a solution of the protected tridecapeptide in trifluoroacetic acid/diethylphosphite/methylethylsulfide 4:1:1[2]. After purification by a countercurrent distribution of 381 transfers (K = 1.6) in the system sec-butanol/trifluoroacetic acid/water 200:1:200, H-Ser-Tyr-Ser-Met-Glu(OH)-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH_2 (= analogue A) was obtained in an homogeneous state (61% yield), as proved by paper chromatography[16] (Rf_M = 0.22. Rf_P = 0.10. Rf_A = 0.25) and paper electrophoresis[16]. ($E_{1.9}$ = 1.1 Glu = 1.4 Try = 0.7 His. $E_{5.8}$ = 0.8 His = 2.1 Try) after revelation by the ninhydrin, bromphenolblue, chlorine-starch-iodide, Folin and Sakaguchi reagents[17]. The tridecapeptide was entirely digested by leucine-aminopeptidase, giving the composing amino acids in the expected ratio. It exhibited 1/15 of the melanophore stimulating activity of α-MSH[8,9], but no unequivocal ACTH activity was detected either *in vitro*[9,18] or *in vivo*[9,19].

Condensation of CBO-Ser-N_3[20] with H-Met-Glu(OBz)-OH[13] afforded CBO-Ser-Met-Glu(OBz)-OH (49% yield. M.p. 116°. $[\alpha]_D^{20}$ = −11° (methanol), −25° (dimethylformamide). Analysis calculated for $C_{28}H_{35}O_9N_3S$: C 57.1; H 6.0; O 24.4; N 7.1; S 5.4. Found: C 57.3; H 5.9; O 24.7; N 6.9; S 5.5). This tripeptide was condensed with H-His-Phe-Arg-Try-Gly-(ε-CBO)Lys-Pro-Val-NH_2[15] by dicyclohexylcarbodiimide in acetonitrile/dimethylformamide 2:1 to CBO-Ser-Met-Glu(OBz)-His-Phe-Arg-Try-Gly-(ε-CBO)Lys-Pro-Val-NH_2 (72% yield. M.p. 178°. Analysis calculated for $C_{86}H_{112}O_{18}N_{19}SCl$: C 58.4; H 6.4; N 15.1; S 1.8. Found: C 58.7; H 6.5; N 15.0; S 1.8. $[\alpha]_D^{23}$ = −36° (methanol), −25° (dimethylformamide)).

Scission of the protecting group of a first portion of the protected endecapeptide was effected by passing a current of dry hydrogen bromide into a solution of this peptide in trifluoroacetic acetic/diethylphosphite/methylethylsulfide 4:1:1[2]. After purification by countercurrent distribution (K = 4.0) in the system sec-butanol/trichloracetic acid/water 200:1:200, H-Ser-Met-Glu(OH)-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH_2 (= analogue B) was obtained in a homogeneous state, as proved by paper electrophoresis ($E_{1.9}$ = 0.7 His = 1.1 Glu = 1.4 Try; $E_{5.8}$ = 0.7 His) after revelation by the ninhydrin, bromphenolblue, chlorine-starch-iodide, Folin and Sakaguchi reagents[17]. The endecapeptide exhibited a melanophore stimulating activity[8] amounting to only 1/100 of that of α-MSH and no unequivocal *in vitro* ACTH activity[18].

Scission of the protecting groups of a second portion of the protected endecapeptide was accomplished by passing a current of dry hydrogen bromide in a solution of this peptide in acetic acid/diethylphosphite/methylethylsulfide 4:1:1. Under these conditions an O-acetylation of the serine residue takes place[13]. After purification by countercurrent distribution (K = 5.2) in the system sec-butanol/trichloracetic acid/water 200:1:200, H-(AcO)Ser-Met-Glu(OH)-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH_2 was obtained in a homogeneous state, as proved by paper electrophoresis[16] ($E_{1.9}$ = 1.1 Glu; $E_{5.8}$ = 0.7 His) after revelation by the ninhydrin, chlorine-starch-iodide, Folin and Sakaguchi reagents[17]. The product was converted in the corresponding *N*-acetyl endecapeptide by a sojourn of 1 h at pH 7.9 ± 0.1. After purification by countercurrent distribution (K = 3,5) in the same system as above, Ac-Ser-Met-Glu(OH)-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH_2 (= analogue C) was obtained in an homogeneous state, as proved by paper electrophoresis[16] ($E_{1.9}$ = 1.0 Glu; $E_{5.8}$ = 0.5 His) after relevation by the ninhydrin, bromphenolblue, chlorine-starch-iodide, Folin and Sakaguchi reagents[17]. The acetylated tridecapeptide exhibited a melanophore stimulating activity[8] amounting to 1/4 of that of α-MSH.

Our experimental results show that the absence of the *N*-acetyl group on the *N*-terminal serine residue of α-MSH (analogue A) brings about a sharp decrease of the melanophore stimulating activity, as had been formerly predicted by HARRIS[21].

As expected, a further decrease of the melanophore stimulating activity results, when not only the acetyl group, but also the two first amino residues of the *N*-terminal extremity of the α-MSH chain are suppressed (analogue B). On the other hand, it is surprising that a

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[13] ST. GUTTMANN and R. A. BOISSONNAS, Helv. chim. Acta *41*, 1852 (1958).
[14] CBO- = carbobenzyloxy-; Bz- = benzyl-; Ac- = acetyl. All the amino acids bearing an asymetric carbon are of the *L* configuration.
[15] R. A. BOISONNAS, ST. GUTTMANN, R. L. HUGUENIN, P. A. JAQUENOUD, and ED. SANDRIN, Helv. chim. Acta *41*, 1867 (1958).
[16] Ascending paper chromatography on Schleicher and Schuell washed paper No. 2040b. Rf_M in methylethylcetone/pyridine/water 65:15:20. Rf_A in isoamylalcohol/pyridine/water 35:35:30. Rf_P in *n*-butanol/acetic acid/water 70:10:20. Paper electrophoresis under high tension (70 V/cm). $E_{1.9}$ at pH 1.9 in formic acid/acetic acid/water 15:10:75. $E_{5.8}$ at pH 5.8 in pyridine/acetic acid/water 9:1:90. $E_{1.9}$ = 1.1 Glu indicates that at pH 1.9 the compound migrates 1.1 time the distance run by glutamic acid.
[17] For the exact composition of the reagents see ref. [2].
[18] The assays were kindly performed by Dr. ZEHNDER of our Pharmacological Department.
[19] A similar observation was recently preliminary reported by K. HOFMANN et al., J. Amer. chem. Soc. *83*, 487 (1961).
[20] J. S. FRUTON, J. biol. Chem. *146*, 463 (1942).
[21] J. I. HARRIS, Symposium on Protein Structure (Methuen & Co., London 1958), p. 333. This conclusion is also in accordance with the recent observation by J.-P. WALLER and B. F. DIXON, Biochem. J. *75*, 320 (1960), that the introduction of an *N*-acetyl group on the *N*-terminal serine residue of ACTH causes a five to ten times increase of the small inherent melanocyte stimulating activity of this hormone.

Influence of the structure of the *N*-terminal extremity on the melanophore stimulating activity

Chemical structure		Relative melanophore stimulating activity (in molar relation to α-MSH)
Ac-Ser-Tyr-Ser-Met-Glu(OH)-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH_2	(α-MSH)	*1*
H-Ser-Tyr-Ser-Met-Glu(OH)-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH_2	(analogue A)	1/15
H-Ser-Met-Glu(OH)-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH_2	(analogue B)	1/100
Ac-Ser-Met-Glu(OH)-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH_2	(analogue C)	1/4

simple acetylation of the *N*-terminal extremity of this only slightly active endecapeptide brings about a twenty five times increase of the melanophore stimulating activity (analogue C).

We see two possible explanations for this phenomenon: either the *N*-terminal structure of the chain has to be Ac-Ser- for the appearance of a strong melanophore stimulating activity, or the presence of an acetyl group at the extremity of the peptide chain ensures its resistance against degradation by aminopeptidases, thus increasing the apparent biological activity of the acetylated molecules by simply lenghtening their life.

Zusammenfassung. Drei strukturelle Analoge des α-MSH wurden synthetisiert und auf ihre Melanophoren-stimulierende Wirksamkeit untersucht. Für das Auftreten einer ausgeprägten biologischen Aktivität scheint die Anwesenheit einer *N*-Acetyl-Gruppe an dem Aminoende der Peptidkette wichtiger zu sein als das Vorhandensein der zwei ersten amino-endständigen Aminosäurereste der α-MSH-Kette.

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Studies on Inhibition of 3,4-Dihydrophenylethylamine (Dopamine) β-Oxidase *in vitro*[1]

The availability of a method for the preparation of purified dopamine β-oxidase[2] makes it possible to study the mechanism of the hydroxylation and the substrate specificity of the enzyme. The hydroxylation of the side chain of dopamine may be the rate-limiting step in the biosynthesis of norepinephrine and, consequently, inhibitors of dopamine hydroxylation may be important therapeutic agents in lowering norepinephrine levels *in vivo*.

This is a preliminary report of a study designed to explore the possibility of structural analogues of dopamine as well as structurally unrelated compounds being inhibitors of dopamine β-oxidase.

The test compounds were added simultaneously with 40 μg dopamine-1-C^{14} to a mixture which contained the following components (in μMol): potassium phosphate buffer, pH 6.4, 100; (1-methyl-2-phenyl)-ethyl hydrazine hydrochloride, 1.3; ascorbic acid, 6; fumaric acid, 10; ATP, 12.5. To this mixture 0.2 ml of the enzyme was added[3] and the final volume was adjusted with phosphate buffer pH 6.4 to 1 ml. The reaction mixture was incubated for 1 h at 37°C using air as a gas phase. At the end of the incubation the reaction was stopped by the addition of 3% acetic acid in ethanol, and the solution heated at 55°C for 5 min. The precipitate proteins were removed by centrifugation. The alcohol in the supernate was evaporated under nitrogen and the solution was analyzed for dopamine-1-C^{14} and norepinephrine-1-C^{14}. Dopamine-1-C^{14} and norepinephrine-1-C^{14} were separated as the acetylated derivatives by paper chromatography in the 'C' solvent system of Bush[4]. The relative inhibition rate of dopamine β-oxidase by each compound was determined by the comparison of the amount of norepinephrine-1-C^{14} formed in an incubation mixture which contained only the substrate and the incubation mixture which contained the compound to be tested and the substrate.

The accompanying Table shows the effects of the test compounds on the conversion of dopamine to norepinephrine. Several generalizations may be deduced from the Table. Primary phenylethylamines and phenylpropylamines are active inhibitors, while secondary amines are less active. Phenolic amines are even more potent inhibitors than phenyl amines, but methoxy amines are less active. The nature of the inhibition and the activity of the inhibitors *in vivo* are under investigation.

Added in proof: It was also found that homarylamine and impramine (Tofranil) are inhibitors of dopamine β-oxidase. We have also been able to show that phenylethylamine, 3-methoxydopamine, and *p*-hydroxyemphetamine are substrates of dopamine β-oxidase and are converted by it to the corresponding β-hydroxy compounds.

Zusammenfassung. Die enzymatische Umwandlung von Dopamin in Norepinephrine wird durch primäre Phenylethylamine und Phenylpropylamine sowie durch Pentobarbital gehemmt.

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Department of Psychiatry and Neurology, New York University, College of Medicine, New York, January 16, 1961.

[1] This work was supported by grants from the National Institutes of Health.

[2] E. Y. LEVIN et al., J. biol. Chem. *235*, 2080 (1960).

[3] The enzyme was prepared by the method of E. Y. LEVIN et al.[2] but the purification on calcium phosphate gel was omitted.

[4] M. GOLDSTEIN et al., Proc. Soc. exp. Biol. Med. *103*, 137 (1960).

Inhibition of dopamine-to-norepinephrine conversion *in vitro*

Active compounds	Amount added in μg	% of Inhibition	Inactive compounds	Amount added in μg	% of inhibition
p-hydroxyphenylethylamine	400	70–80	N-acetyl dopamine	400	0
β-phenylethylamine	400	40–50	3,4-dihydroxyphenylethylmethylamine	400	0–5
dl-d-methylphenethylamine (Amphetamine)	800	30–40	(Epinine)		
2-phenylcyclopropylamine (SKF-385)	800	30–40	3-methoxy-4-hydroxyphenylethylamine	400	0–5
Pentobarbital sodium	800	20–30	(3-methoxydopamine)		
3,4-dihydroxyphenylethylamine (Epinine)	800	10–20	3,4,5-trimethoxyphenylethylamine (Mescaline)	400	0
3-methoxy-4-hydroxyphenylethylamine	800	10–20	N,N-diethyl-*d*-lysergamide (LSD-25)	400	0
(3-methoxydopamine)			Phenylmethylaminopropane (*d*-Desoxyephedrine)	400	0–5