197. Tritiation of Peptides to High Specific Radioactivity

Part 1

Synthesis and Biological Properties of [13-($^3H_4$)Norvaline]-α-MSH and of [2,23-Bis(($^3H_2$)tyrosine)]ACTH(1–24)[1])

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Dedicated to Professor *Max Brenner*, Basel, on the occasion of his 70th birthday

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α-MSH and ACTH(1–24) were tritiated to high specific radioactivity (> 100 Ci/mmol) using a new tritiation apparatus with which the tritiation reaction can be performed at slightly elevated pressure. This allows short reaction times with the least possible damage to the molecule. The starting compounds for the tritiation were [13-propargylglycine]α-MSH and [2,23-Bis(3′,5′-diiodotyrosine)]ACTH(1–24). Both tritiations were quantitative and yielded products of high purity, full biological activity, and with a specific radioactivity of 115 Ci/mmol and 100 Ci/mmol, respectively.

Introduction. – The study of receptors for α-melanotropin (α-MSH) and adrenocorticotropin (ACTH) on adrenal cells, pigment cells, and in the brain requires biologically active hormones of high specific radioactivity. ^{125}I-Labelling has proved useful for α-MSH since the iodinated peptide retains at least 50% of the activity of the parent hormone when tested in pigment-cell assays [2]. In contrast, iodination of native ACTH(1–39) or ACTH(1–24) at the tyrosine residue[2] – the predominant reaction during this type of labelling – results in a dramatic loss of potency [3]. Comparative studies of the interaction of labelled α-MSH and ACTH with a particular target tissue such as the adrenal cortex necessitates the insertion of the same radioactive label, preferably into the native structure of the hormone such that the biological and physicochemical properties of the two peptides are not altered. This can only be achieved by using ^{3}H as radioactive label since chemical synthesis with (^{35}S)methionine is too expensive and with (^{14}C)amino acids does not produce sufficiently high specific radioactivity. We have, therefore, investigated the preparation of tritiated α-MSH and ACTH(1–24) containing > 100 Ci of ^{3}H per mmol of hormone.

[1]) Parts of this report have been presented at the 9th American Peptide Symposium, Toronto, June, 1985. Nomenclature and abbreviations, see [1]. Additional abbreviations: HMPTA = hexamethylphosphoric triamide; ACTH = adrenocorticotropin; MSH = melanotropin (melanocyte-stimulating hormone); Msoc = 2-(methylsulfonyl)ethoxycarbonyl; Pap = 4-azidophenylalanine; Pra = propargylglycine. All chiral amino acids are in the L-configuration.

In order to obtain labelled hormones with this high specific radioactivity, at least four ^{3}H-atoms have to be inserted into each peptide molecule, either by catalytic hydrogenation of a triple bond or by catalytic halogen/^{3}H exchange on two tyrosine and/or histidine residues carrying two I- or Br-atoms. The latter approach was studied by *Brundish et al.* [4] who prepared [2,23-bis(3′,5′-diiodotyrosine)]ACTH(1–24) as precursor, and the former was first tested in a preliminary experiment with α-MSH [5] containing a propargylglycine residue. The conditions used in earlier tritiation experiments (usually 10 Ci, *i.e.* 4 ml, of $^3H_2$ gas [4] [6–8]) were such that the initial gas pressure was considerably below 1000 mbar and was further reduced as the reaction proceeded. As a consequence, the reaction never went to completion, and the products had to be extensively purified in order to remove varying quantities of starting product. In contrast, hydrogenation experiments at slightly elevated pressure with the same precursors showed that the desired products were formed quantitatively and in a very short time. For this reason, we have investigated a new tritiation apparatus with which $^3H_2$-gas pressures of up to 1650 mbar can be applied to the peptide solutions. This paper describes in detail the apparatus and the labelling procedure of the two model peptides [Nva($^3H_4$)^{13}]-α-MSH and [Tyr($^3H_2$)^{2,23}]ACTH(1–24), including the synthesis of the precursor for tritiated α-MSH, [Pra^{13}]-α-MSH, and of the non-radioactive analogue [Nva^{13}]-α-MSH. The preparation of peptides with up to 8 ^{3}H-atoms per molecule (*i.e.* > 200 Ci/mmol) will be presented in two forthcoming publications.

Synthesis of α-MSH Derivatives. – The structures of α-MSH (**I**) and ACTH (**II**) are depicted in *Fig. 1*, together with the modified residues used for insertion of ^{3}H. The reason for labelling α-MSH at the C-terminus was the need for a derivative carrying the label in the second hormonally active site [9]; in an earlier tritiation experiment with α-MSH [2], the radioactivity was incorporated into Tyr-2, *i.e.* near the N-terminus. For the present synthesis, the C-terminal valine was replaced by propargylglycine (Pra) yielding norvaline (2-aminopentanoic acid, Nva) upon tritiation. This alteration was expected to have little influence on the biological activity of the hormone since previous studies [10] [11] have shown that derivatives with even less similarity to the natural peptide, such as [Met^{13}]-α-MSH or [Pap^{13}]-α-MSH, retained a good potency. Another advantage of using Pra is the fact that ^{3}H incorporated into the side-chain of an aliphatic amino acid remains firmly attached even after boiling in strong acid whereas in aromatic residues it is easily exchanged.

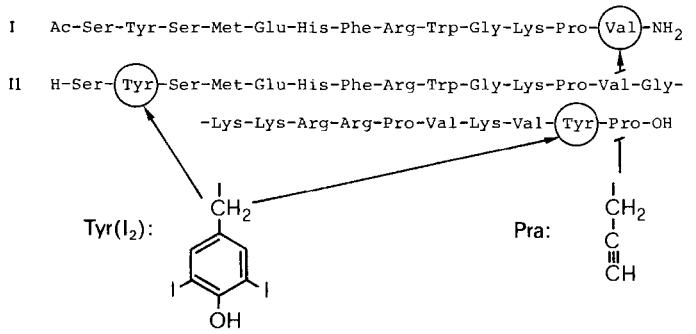
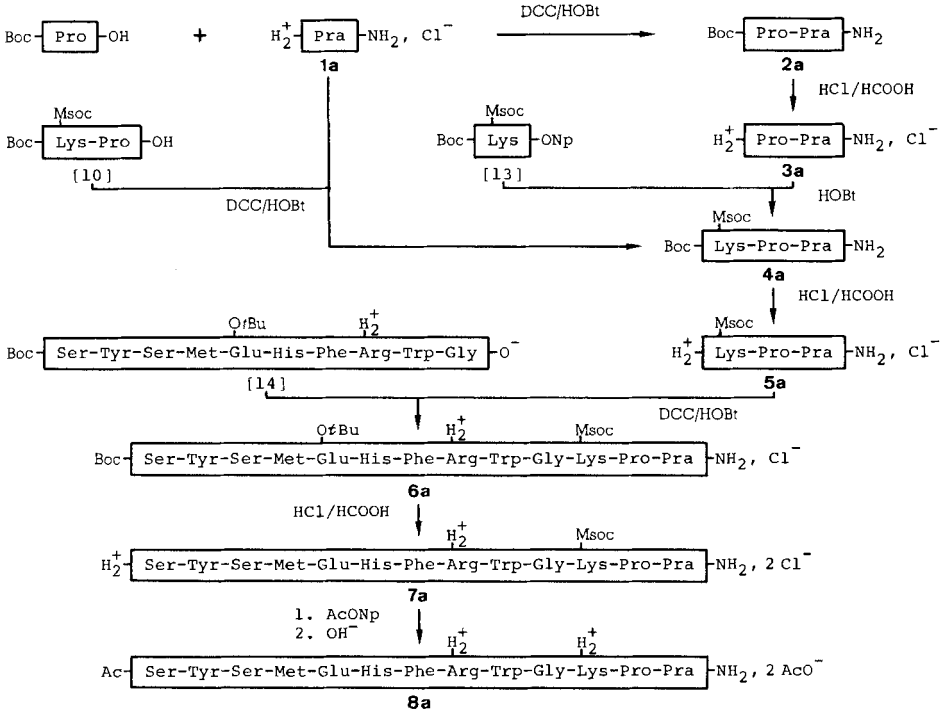


Fig. 1. *Structure of α-MSH* (**I**) *and of ACTH(1–24)* (**II**) *with the corresponding modifications for tritium insertion*

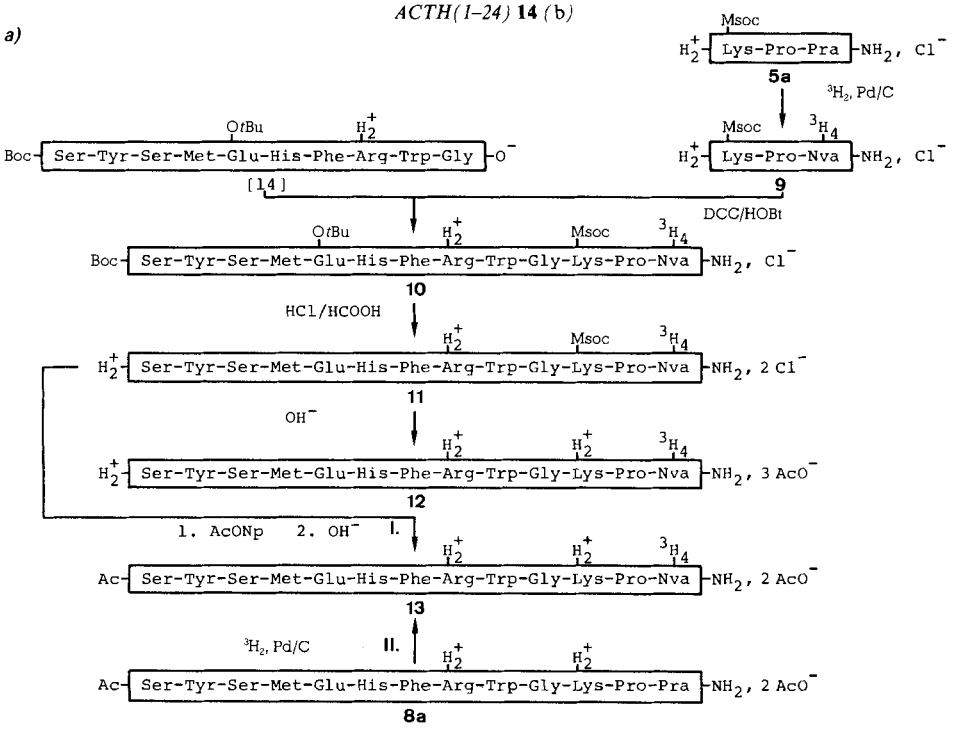
Scheme 1. *Synthesis of [Pra[13]]-α-MSH·2AcOH* (**8a**) *and of [Nva[13]]-α-MSH·2AcOH* (**8b**; Pra in **8a** is replaced by Nva)



The precursor for the tritiation, [Pra[13]]-α-MSH·2 AcOH (**8a**), and its reference compound, [Nva[13]]-α-MSH·2 AcOH (**8b**), were synthesized from **1** and **2** in the same way by a classical approach in homogeneous solution, following the strategy of earlier syntheses of α-MSH [10–13]. The intermediates **3**–**7** were, whenever possible or desirable, isolated, purified, and characterized. As outlined in *Scheme 1*, the Boc/O*t*Bu-protected decapeptide sequence (1–10) [14] was coupled to the tripeptide sequence (11–13), H-Lys(Msoc)-Pro-Pra-NH_2·HCl (**5a**) or H-Lys(Msoc)-Pro-Nva-NH_2·HCl (**5b**), using the DCC/HOBt method of *König* and *Geiger* [15]. The acid-labile butyl-type protecting groups of **6** were removed by treatment with 0.1N HCl in HCOOH (→**7**), and the free N-terminal α-amino group was acetylated with *p*-nitrophenyl acetate. Finally, the base-labile Msoc-protecting group was cleaved with 0.1N NaOH/DMF/MeOH 2:1:1, and the product was purified by chromatography on carboxymethyl-cellulose and *Sephadex LH-20*. Analysis of [Pra[13]]-α-MSH·2 AcOH (**8a**) and [Nva[13]]-α-MSH·2 AcOH (**8b**) by HPLC, TLC, thin-layer electrophoresis (TLE), and amino-acid analysis showed that they were pure and contained the correct amino-acid composition.

Since it has been shown with ACTH [7] and α-MSH [2] that tritiations of precursors containing the entire amino-acid sequence lead to unspecific insertion of 8–10% of ^3H into additional residues, the (11–13) tripeptide fragment **5a** itself was tritiated in a second experiment to yield radioactive **9** which was then coupled to the (1–10) sequence *(Scheme 2)*. Deprotection of **10** (→**11**) and acetylation (→**13I**) were identical to the experiment

Scheme 2. *Synthesis of Tritiated [Nva[13]]-α-MSH* **13** *and Des-acetyl-[Nva[13]]-α-MSH* **12** *(a) and of Tritiated ACTH(1–24)* **14** *(b)*



describing the non-radioactive analogue [Nva[13]]-α-MSH · 2 AcOH (**8b**). This more laborious approach also allowed a straightforward preparation of tritiated des-acetyl-[Nva[13]]-α-MSH **12** and, when compared with the first synthesis (→**13II**), made it possible to determine exactly the extent of undesired incorporation of ^3H that occurred during tritiation in the new apparatus and, respectively, during exposure to the various conditions used in peptide synthesis.

The precursor for the tritiation of ACTH(1–24) was [Tyr(I$_2$)^{2,23}]ACTH(1–24). The compound was synthesized by *Brundish et al.* [4] by a classical solution approach and kindly made available to us.

Tritiation Apparatus. – The main features of the $^3H_2$-handling unit are the use of uranium traps for storing the $^3H_2$ gas and the construction of a fully metal-sealed manifold system made of welded stainless steel (series 300) in order to obtain high resistance and low permeability to H_2. This also allows elimination of any solvent traces and of other impurities from the system by heating it with a welding-torch to high temperature. Uranium traps offer the advantage that contaminating He can readily be removed. In addition, they have a high $^3H_2$-binding capacity (in the form of uranium tritide, $^{238}U^3H_3$) and a low $^3H_2$-dissociation pressure at the storage temperature: the equilibrium pressure is about 2.5×10^{-3} mbar at 20°C and 1000 mbar at 400°C [16]. Thus, the $^3H_2$-gas pressure can easily be adjusted by increasing (or decreasing) the temperature of the uranium trap, resulting in a pressure range of this unit from a few mbar up to 1650 mbar.

The different parts of the tritiation apparatus are depicted in *Fig. 2:* The reaction vessel (RV; *Pyrex* glass) is coupled to the unit by *Swagelock PTFE* ferrules. The solvent trap (ST) is mounted after the tritiation when the solvent from the reaction vessel has to be distilled off. A valve (V1) separates this distillation arc from the rest of the

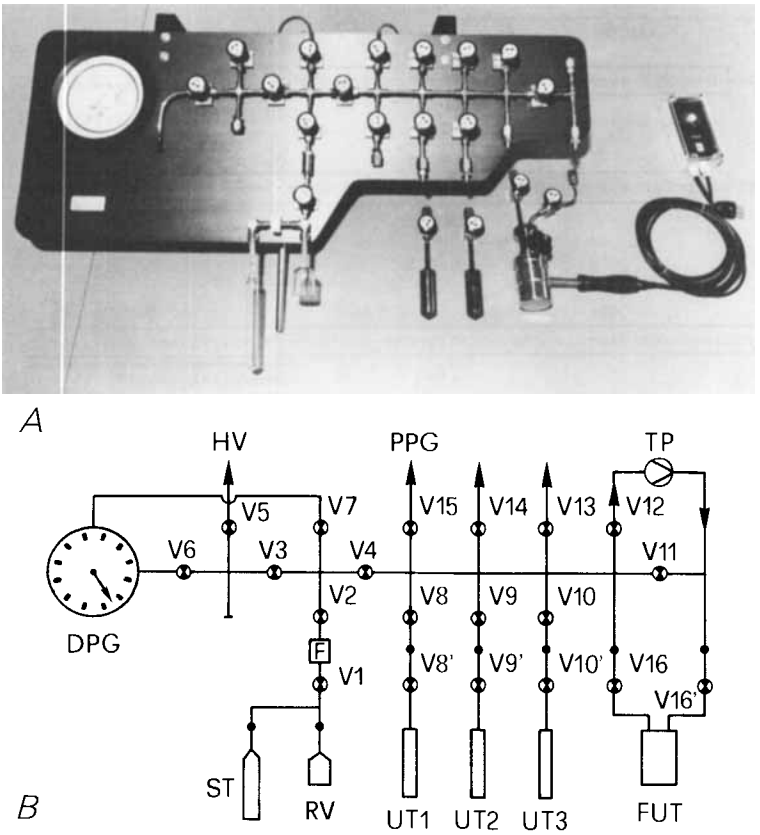


Fig. 2. *Foto (A) and scheme (B) of the tritiation apparatus.* DPG: differential pressure gauge; F: filter; FUT: flow-through uranium trap; HV: high vacuum; PPG: *Pirani* pressure gauge; RV: reaction vessel; ST: solvent trap; TP: *Toepler* pump; UT: uranium trap; V: valve (for further details see text).

unit. A filter (F) connects V1 with V2. Valves V3 and V4 connect the RV line either to the high vacuum or to the $^3H_2$ source. V5 leads to the high vacuum (*Pfeiffer* turbo molecular pump *TPH 100* for oil-free vacuum). The differential pressure gauge (DPG; *Model D-62A*, series *1500*, *Wallace & Tierman*, Gürnzburg, FRG) is connected *via* V6 and V7 and measures the $^3H_2$-gas pressure within the unit during the experiment. V8, V9, and V10 connect one-valve (V8', V9', V10') uranium traps (UT). These are made at *Radium-Chemie (A.Z.)* from stainless steel (*SS 316*, wall thickness 3 mm) and loaded with uranium-238 pyrophoric powder in such a way that channelling of the gas through the bed is prevented. The capacity of these traps ranges from 1000 to 25000 Ci of $^3H_2$. A flow-through uranium trap (FUT) serves to recover $^3H_2$ of lower purity. The flow is generated by a Hg-containing *Toepler* pump (TP) with which traces of gas in the unit can be recirculated through the FUT. Valves V13 and V14 lead to either $^3H_2$ tanks (for large-scale reactions) or external gas supply (such as H_2, N_2, Ar). Finally, V15 connects the system to the *Pirani* pressure gauge (PPG; *Balzers TPR 010*) with which the initial vacuum is controlled. The volume of the unit (without reaction vessel and extra $^3H_2$ tank) is about 30 ml. When the tritiation is performed in a 50-ml reaction vessel, the $^3H_2$-gas volume is about 75 ml (80 ml minus volume of solvent, usually 5–6 ml). This means that at the maximal gas pressure of this unit of 1650 mbar (1250 Torr), the total ^3H content in the system reaches about 300 Ci so that the tritiation proceeds at almost constant pressure. (For small-scale preparations, a 25-ml reaction flask can be used which reduces the total volume of the $^3H_2$ to 50 ml, *i.e.* 200 Ci.)

The apparatus is mounted in upright position in a completely closed glove-box, the pressure of which is held at minus 50 mm H_2O. The air is permanently monitored with a $^3H_2$ detector (*Triton model 955B, Johnston Inc.*, Cockeysville MD), and the exhaust air from the vacuum pump is circulated through a catalytic converting system which oxidizes any traces of $^3H_2$ to H^3HO. The latter is adsorbed onto a molecular sieve.

Tritiation of α-MSH and ACTH Derivatives. – Catalytic tritiation of the triple bond of Pra (compounds **5a** and **8a**; *Scheme 2*) was performed in the presence of 10% Pd/C, whereas for the catalytic dehalogenation of protected [Tyr$(I_2)^{2,23}$]ACTH(1–24), a 1:1 mixture of 5% Pd/C and 5% Rh/$CaCO_3$ [6] was used. The initial $^3H_2$-gas pressure was usually 1620–1650 mbar which decreased slightly as the reaction proceeded. The uptake of $^3H_2$ was complete within 5 min; the reaction was continued for another 10 min in order to obtain quantitative tritiation of the starting product. This relatively short exposure to high doses of $^3H_2$ minimized the formation of by-products, as shown later during purification and analysis of the peptides.

The incorporation of ^3H into the peptide and the solvent/catalyst could be calculated from the pressure difference between start and end of the reaction since 1 Torr corresponded to about 0.25 Ci of $^3H_2$-gas. In all three reactions, the ^3H content of the reaction mixture was higher than that specifically incorporated into the peptide: 3.5 Ci for **9** (20 μmol peptide→maximal specific incorporation: 2.36 Ci), 1.75 Ci for **13II** (5 μmol peptide→0.59 Ci), and 1.25 Ci for **14** (1 μmol peptide→0.12 Ci). Some of the excess ^3H could be accounted for by exchanged amide H-atoms in the peptide, but most, usually about 1 to 1.5 Ci, was contained in the solvent/catalyst. This excess was removed (1) by repetitive thawing and freezing of the reaction mixture and simultanous pumping with the *Toepler* pump of liberated gas through the FUT and (2) by repetitive distillation of the solvent and of readded DMF/H_2O/EtOH from RV into ST. This procedure followed by an additional cycle of repetitive concentration/redilution of the product (after the catalyst had been centrifuged off) allowed virtually all 'loosely' bound ^3H to be exchanged in the safest possible way. However, part of the peptide, in this experiment particularly the unprotected [Nva$(^3H_4)^{13}$]-α-MSH, was lost by adsorption to the catalyst, and hence the overall yield was considerably lower (for **13II** almost by a factor of 2) than in the parallel experiment with H_2 and straight centrifugation of the catalyst.

Tritiated **9** was obtained in 65% yield after purification by precipitation. The precipitate was coupled to the protected (1–10) fragment **10** *(Scheme 2a)* and the resulting tridecapeptide **11** further processed as described for [Nva^{13}]-α-MSH · 2 AcOH (**8b**). The

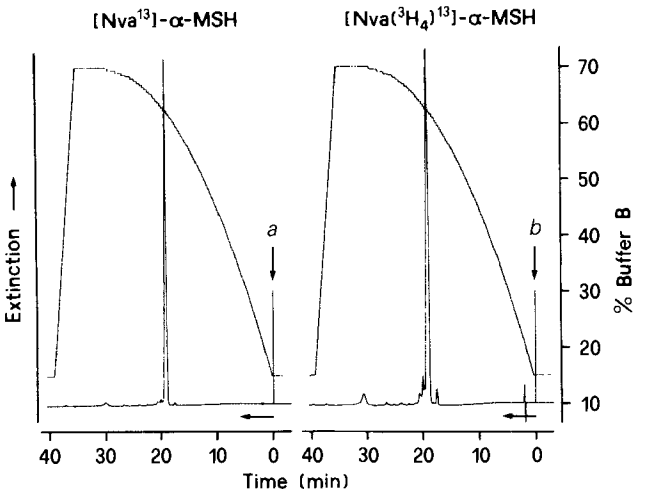


Fig. 3. *HPLC of [Nva^{13}]-α-MSH* **8b** *(a) and its tritiated analogue* **13** *(b)*. Injection: 50 μg of peptide. The small peak at *t* 17.6 min (*b*) is the Met(0) derivative. The gradient tracing of buffer B runs two min earlier than the peptide elution. For details see *Exper. Part*.

overall yield from the tritiation of **5a** to the final product **13I** was 29%. This yield was only slightly lower than that of the direct tritiation of **8a** to **13II** (35%), mainly because of smaller losses by adsorption to the catalyst of the tripeptide **9** as compared to those of the tridecapeptide **13II** (see above). In both cases, the product was obtained in pure form after a single filtration over *Sephadex LH-20* (see *Fig. 3*), and the specific radioactivity was 115 Ci/mmol. [Tyr($^3H_2$)^{2,23}]ACTH(1–24)·8 AcOH (**14**) was obtained in 45% yield after cleavage of Boc/O*t*Bu and repetitive (2 ×) filtration over *Sephadex LH-20*. The higher yield relative to [Nva($^3H_4$)^{13}]-α-MSH was due to the presence of protecting groups during exposure to the catalyst *(Scheme 2b)*. The specific radioactivity was 100 Ci/mmol.

Comparison of the ^3H content of the chymotryptic fragments [2] of **13I** and **13II** showed that direct tritiation of [Pra^{13}]-α-MSH **8a** produced about 7% non-specific insertion whereas tritiation of the tripeptide (11–13) **5a** and subsequent condensation with the (1–10) fragment decreased the non-specific insertion to $< 0.5\%$ (see *Exper. Part*). This finding corresponds with earlier reports concerning non-specific insertion of ^3H into α-MSH [2] and ACTH [7]. It also shows that non-specific insertion is not increased by tritiation at elevated pressure when compared with earlier tritiations at reduced pressure.

Biological Activities. – The melanotropic activities of the radioactive peptides and the corresponding non-radioactive reference compounds in three pigment-cell assays are displayed in the *Table*. The results show that changing Val-13 of α-MSH into Nva-13 or Pra-13 did not alter the biological activity of the peptide in any of the three pigment-cell systems. As expected, the tritiated peptide [Nva($^3H_4$)^{13}]-α-MSH **13** retained full melanotropic activity in all three assays. These results strengthen earlier observations that the C-terminal residue of α-MSH is not critical for a good potency of the hormone and may, therefore, serve as site for introducing labels into the peptide. Des-acetyl-[Nva($^3H_4$)^{13}]-α-MSH **12** exhibited the same potency as des-acetyl-α-MSH, *i.e.* between 8% and 30%, depending on the assay.

Table. *Melanotropic Activities of Tritiated α-MSH Derivatives and ACTH(1–24) and of Non-radioactive Reference Compounds*

Compound	Pigment dispersion[a] (Units/mmol)		Tyrosinase stimulation[b] ($1/EC_{50} \times 100$)
	Rana pipiens	*Anolis carolinensis*	*Cloudman* mouse melanoma cells
α-MSH	4×10^{10}	4×10^{10}	4×10^{10}
Des-acetyl-α-MSH	3×10^{9}	1.2×10^{10}	1×10^{10}
[Pra^{13}]-α-MSH **8a**	4×10^{10}	3×10^{10}	4×10^{10}
[Nva^{13}]-α-MSH **8b**	4×10^{10}	4×10^{10}	4×10^{10}
Des-acetyl-[Nva($^{3}H_4$)^{13}]-α-MSH **12**	3×10^{9}	1×10^{10}	1×10^{10}
[Nva($^{3}H_4$)^{13}]-α-MSH **13**	4×10^{10}	4×10^{10}	4×10^{10}
[Tyr($^{3}H_2$)^{2,23}]ACTH(1–24) **14**	–	5×10^{8}	–

[a]) Pigment-dispersing potencies, determined with the modified *in vitro Rana pipiens* [9] and the *Anolis carolinensis* [18] skin assay, are shown in units/mmol which is equivalent to $1/EC_{50}$ *(Rana)* and $1(EC_{50} \times 8$ *(Anolis)*.

[b]) Tyrosinase-stimulating potencies, measured with *Cloudman S-91* mouse melanoma cells in culture, are expressed as relative molar potencies ($1(EC_{50} \times 100)$.

The steroidogenic potency of tritiated ACTH(1–24) **14** was determined with an *in vitro* assay using isolated cells from the inner zones of the rat adrenal cortex [17]. At a hormone concentration of 1.4×10^{-11}M, the stimulation with standard ACTH(1–24) *(Synacthen)* was maximal (defined as 100%) whereas with the tritiated compound **14** it was 90%. Although this is slightly submaximal when compared with the standard, the log-dose/response curves (to be published elsewhere) are almost identical and hence the peptides almost equipotent. This means that despite the high ^{3}H content during the reaction, oxidation at crucial residues such as at Trp-9 or Met-4 has not occurred or only in such small quantities that they were easily removed during a simple gel filtration. The steroidogenic activity of tritiated [Nva^{13}]-α-MSH was not determined since normal α-MSH retains only about 0.01% of the activity of ACTH(1–24) in this assay [17].

Discussion. – This report shows that both tritiation of a triple bond and halogen/^{3}H exchange proceed quickly and quantitatively even in the presence of methionine when the reaction is performed at elevated and constant $^{3}H_2$-gas pressure. The short exposure time to high concentrations of $^{3}H_2$ gas did not lead to noticable radiation damage; pure peptides were obtained after simple gel filtration of the final products. The specific radioactivity incorporated into [Nva^{13}]-α-MSH (115 Ci/mmol) almost reached the maximal value of 118 Ci/mmol, whereas ^{3}H content in ACTH was slightly lower (100 Ci/mmol), most probably due to exchange with amide or side-chain H-atoms during the tritiation or with protons during cleavage of Boc/O*t*Bu.

As expected for the tritiation of the [Pra^{13}]-α-MSH tridecapeptide, the *Wilzbach* effect [19] produced considerable non-specific incorporation of ^{3}H into residues other than Pra-13 because aromatic side chains (in particular the imidazole ring of His-6) may undergo $^{1}H/^{3}H$ exchange relatively easily. Non-specific incorporation was however negligible when the (11–13) fragment was tritiated before condensation to the (1–10) fragment. For most studies such a high degree of specificity is not usually required except when breakdown or uptake by the target tissue is examined.

The tritiation apparatus described in this report has a wide range of applications: the gas pressure can be chosen from a few mbar up to 1650 mbar and the amount of ^{3}H used

from a few Ci to several kilo Ci. This wide range does not limit the apparatus to 'small-scale' preparations as described here, but also makes it a versatile instrument for tritiations at a commercial scale (*e.g.* production of tritiated luminescent compounds for time pieces). Furthermore, the use of uranium traps for $^3H_2$-gas storage, their safe handling, and the resulting excellent environmental protection make it possible to perform several tritiations on one and the same day.

We wish to express our gratitude to Professor Dr. *J. Girard* for encouragement and support, to Dr. *J.B. Baumann* and Mrs. *E. Christen* for performing the adrenal cell assay and to Mrs. *V. Jäggin, R. Drozdz,* and *C. Schulthess* for the amino-acid analyses. We also wish to thank Mr. *Willy Hübscher* (present address: *Veterinaria AG,* Zürich) for excellent technical assistance during some of the preliminary experiments. Furthermore, we would like to thank Drs. *R. Wade* and *D. Brundish* (*Ciba-Geigy,* Horsham, U.K.) for the generous gift of [2,23-bis(diiodotyrosine)]ACTH(1–24) derivative and for the $Rh/CaCO_3$ catalyst, and Dr. *R. Andreatta* (*Ciba-Geigy,* Basel) for synthetic ACTH(1–10) and ACTH(1–24). Part of this study was supported by the *Swiss National Science Foundation.* Financial aid by the *Ciba Foundation* and the *Sandoz Research Foundation* is gratefully acknowledged.

Experimental Part

Peptide Synthesis. – *General Remarks.* All solvents and reagents were obtained from *Fluka* or *Merck* and were of anal. grade. TLC: *Merck* silica-gel plates *(G 60, F 254)* using the following solvent systems (*v/v*): BAW1 = 2-BuOH/AcOH/H_2O 72:7:21, BAW2 = 1-BuOH/AcOH/H_2O 67:10:23, BPAW1 = 1-BuOH/pyridine/AcOH/H_2O 50:12:12:25, BPAW2 = 1-BuOH/pyridine/AcOH/H_2O 42:24:4:30, BNH_3 = 2-BuOH/3% NH_3 100:44, CMA = $CHCl_3$/MeOH/AcOH 95:5:3. Thin-layer electrophoresis (TLE): *Merck* cellulose plates using an apparatus of *Camag* (1 h at 25 V/cm) and the following buffers: pH 3.6 = AcOH/pyridine/H_2O 100:10:890, pH 5.6 = AcOH/pyridine/H_2O 6:23:970. The compound spots on TLC and TLE plates were detected by autoradiography (radioactive compounds) and with I_2 vapour, ninhydrin, and *Reindel-Hoppe* [20] reagents. For the quantitative determination of the ^3H content of the 4 chymotryptic fragments on TLC, the compound spots were eluted and counted in the beta-counter (see below). HPLC: *Dupont 8800* instrument equipped with a *Shandon ODS* 5 μm/4 × 250 mm anal. column; flow rate 1.2 ml/min using the buffer systems 0.1% aq. CF_3COOH (A) and CF_3COOH (0.1%)/H_2O (30%)/CH_3CN (70%) (B) in the following composition: 15% B (2 min), exponential gradient from 15 to 85% B (30 min), 85% B (5 min), linear gradient from 85 to 15% B (4 min). Radioactive samples were counted in a *Kontron MR 300* automatic beta-counter. M.p.: in open capillaries with a *Büchi-510* instrument; uncorrected. $[\alpha]_D$: *Perkin-Elmer-141* polarimeter. UV: λ_{max} in nm; *Kontron Uvikon 820* spectrophotometer. Microanalyses were performed in the Institute of Organic Chemistry, University of Basel (Mr. *E. Thommen*), and in the Laboratory of Organic Chemistry, ETHZ (Mr. *D. Manser*). Amino-acid analyses were carried out in our laboratory on a *Waters*-HPLC amino-acid analyser, using the *PicoTag* method. The samples were hydrolyzed in constant boiling HCl containing 1% thioglycolic acid, reacted with phenyl isothiocyanate, and separated on a reverse-phase *C18-PicoTag* column.

H-Pra-NH_2·HCl (**1a**). A suspension of L-propargylglycine [21] (7.48 g, 50 mmol) in MeOH (50 ml) was treated with $SOCl_2$ (4 ml, 55 mmol) at −10°, slowly warmed up to 20°, and kept at 20° for 3 days (TLC (R_f 0.54, BPAW1): quant. reaction). The soln. was evaporated and the residue dried under high vacuum. The methyl ester of Pra (8.18 g, 50 mmol) was dissolved in MeOH (100 ml), treated with liq. NH_3 (75 ml), and kept at 22° for 2 days. The solvent was evaporated, the residue chromatographed on *Amberlyst A-21* with MeOH, and the pure fractions pooled and crystallized from MeOH/Et_2O as amorphous white powder (**1aI**). Recrystallization from MeOH/(i-Pr)$_2O$ yielded 4.65 g (63%) of **1aII** as homogeneous shiny plate-like crystals. M.p. 76° (**1aII**), 224–225° (dec.; **1aI**). TLC: R_f 0.39 (BPAW1), 0.37 (BNH_3). $[\alpha]_D^{25} = -16.7°$ (*c* = 1, MeOH; **1aI**), $[\alpha]_D^{20} = -24.0°$ (*c* = 0.2, MeOH; **1aII**). Anal. calc. for $C_5H_9ClN_2O$ (148.59): C 40.42, H 6.11, N 18.85; found: C 40.23, H 6.28, N 18.55.

H-Nva-NH_2·HCl (**1b**) was prepared in almost the same way and was obtained in pure form. Yield 75%. M.p. 232–233°. TLC: R_f 0.42 (BNH_3), 0.67 (BPAW1).

Boc-Pro-Pra-NH_2 (**2a**). A soln. of Boc-Pro-OH (2.15 g, 10 mmol), **1a** (1.46 g, 10 mmol), and *N*-ethylmorpholine (1.26 ml, 10 mmol) in DMF (40 ml) was treated with HOBt (2.70 g, 20 mmol) and DCC (2.27 g, 11 mmol) at 0° and kept at 0° for 2 h and then at 22° for 36 h. After addition of a few drops of AcOH, the DCU was filtered off and the solvent evaporated. The residue dissolved in EtOAc was consecutively extracted 3 × with 5% $KHSO_4$ soln./5% K_2SO_4 soln. 1:2, 5% aq. $NaHCO_3$ soln., and sat. NaCl soln. The org. solvent was dried with anh.

Na_2SO_4, filtered, and evaporated *in vacuo*. The product was crystallized from benzene/petroleum ether yielding 2.56 g (83%) of pure **2a**. M.p. 72–77°. TLC: R_f 0.60 (BAW1), 0.72 (BPAW1). $[\alpha]_D^{25} = -62.3°$ ($c = 0.56$, MeOH). Anal. calc. for $C_{15}H_{23}N_3O_4$ (309.37): C 58.24, H 7.49, N 13.58; found: C 58.05, H 7.43, N 13.75.

Boc-Pro-Nva-NH$_2$ (**2b**) was obtained in the same way as **2a**. Crystallization from EtOAc/petroleum ether gave pure **2b** in 82% yield. M.p. 127–128°. TLC: R_f 0.68 (BPAW1), 0.70 (BAW1). $[\alpha]_D^{25} = -60.0°$ ($c = 1$, MeOH). Anal. calc. for $C_{15}H_{27}N_3O_4$ (313.40): C 57.49, H 8.68, N 13.41; found: C 57.69, H 8.88, N 13.32.

H-Pro-Pra-NH$_2$·HCl (**3a**). A soln. of **2a** (2.32 g, 7.5 mmol) in 0.12N HCl/HCOOH (75 ml) was kept at r.t. for 10 min and evaporated. The residue was treated with MeOH and evaporated (3 ×) and then crystallized from MeOH/Et_2O: 1.56 g (85%) of pure **3a**. M.p. 181–183°. TLC: R_f 0.51 (BPAW1), 0.60 (BPAW2). $[\alpha]_D^{25} = -42.8°$ ($c = 0.5$, MeOH). Anal. calc. for $C_{10}H_{16}ClN_3O_2$ (245.71): C 48.88, H 6.56, N 17.10; found: C 48.75, H 6.56, N 16.81.

H-Pro-Nva-NH$_2$·HCl (**3b**) was synthesized as described for **3a**. Crystallization from MeOH/Et_2O yielded pure **3b** in 98% yield. M.p. 201–203° (dec.). TLC: R_f 0.19 (BAW1), 0.50 (BPAW1), 0.63 (BPAW2). $[\alpha]_D^{25} = -52.2°$ ($c = 1$, MeOH). Anal. calc. for $C_{10}H_{20}ClN_3O_2$ (249.74): C 48.09, H 8.07, N 16.83; found: C 48.01, H 8.21, N 16.61.

Boc-Lys(Msoc)-Pro-Pra-NH$_2$ (**4a**). a) A soln. of Boc-Lys(Msoc)-ONp [13] (2.59 g, 5 mmol), **3a** (1.23 g, 5 mmol, *N*-ethylmorpholine (0.62 ml, 5 mmol) in DMF (35 ml) was treated with HOBt (0.68 g, 5 mmol) and kept at 20° for 20 h. The solvent was evaporated and the product isolated in EtOAc as described for **2a**. The crude product was then further purified on silica gel with $CHCl_3$/MeOH 9:1. Crystallization from i-PrOH/$(i\text{-Pr})_2O$ gave 2.44 g (83%) of homogeneous **4a**. M.p. 84–86°. TLC: R_f 0.55 (BAW1), 0.64 (BPAW1). $[\alpha]_D^{25} = -46.6°$ ($c = 0.5$, MeOH). Anal. calc. for $C_{25}H_{41}N_5O_9S$ (587.70): C 51.09, H 7.03, N 11.92; found: C 50.89, H 6.99, N 11.60.

b) Similarly, **4a** was obtained by condensation of *Boc-Lys(Msoc)-Pro-OH* (986 mg, 2 mmol) [10] in DMF (15 ml) with **1a** (297 mg, 2 mmol) in the presence of DCC (452 mg, 2.2 mmol), HOBt (540 mg, 4 mmol), and *N*-ethylmorpholine (230 mg, 2 mmol). The product was isolated as described in a): 893 mg (76%). Anal. data as in *a*).

Boc-Lys(Msoc)-Pro-Nva-NH$_2$ (**4b**) was prepared as described for **4a**, except that the crude product was not extracted but filtered over an *Amberlyst 15*/*Amberlyst A21* mixed-bed column with MeOH. Crystallization from i-PrOH/$(i\text{-Pr})_2O$ yielded 80% of pure **4b**. M.p. 71–73°. TLC: R_f 0.53 (BAW1), 0.70 (BPAW1). $[\alpha]_D^{25} = -49.8°$ ($c = 0.57$, MeOH). Anal. calc. for $C_{25}H_{45}N_5O_9S$ (591.73): C 50.75, H 7.67, N 11.84; found: C 50.58, H 7.56, N 11.60.

H-Lys(Msoc)-Pro-Pra-NH$_2$·HCl (**5a**). A soln. of **4a** (0.59 g, 1 mmol) was dissolved in 0.12N HCl/HCOOH (10 ml) and kept at 22° for 10 min. The solvent was evaporated and the residue treated with MeOH (3 ×) and reevaporated. Crystallization from MeOH/Et_2O yielded 0.48 g (92%) of pure **5a**. M.p. 91–96°. TLC: R_f 0.12 (BAW1), 0.38 (BPAW2), 0.40 (BPAW1). $[\alpha]_D^{25} = -39.2°$ ($c = 0.5$, MeOH). Anal. calc. for $C_{20}H_{34}ClN_5O_7S$ (524.05): C 45.84, H 6.54, N 13.36, S 6.12; found: C 45.59, H 6.51, N 12.96, S 6.20.

H-Lys(Msoc)-Pro-Nva-NH$_2$·HCl (**5b**) was prepared as described for **5a** and crystallized from MeOH/Et_2O. Yield 88%. M.p. 115–120°. TLC: R_f 0.45 (BPAW1), 0.56 (BPAW2). $[\alpha]_D^{25} = -37.4°$ ($c = 0.5$, MeOH). Anal. calc. for $C_{20}H_{38}ClN_5O_7S$ (528.07): C 45.49, H 7.25, N 13.26; found: C 45.29, H 7.48, N 12.96.

Boc-Ser-Tyr-Ser-Met-Glu(OtBu)-His-Phe-Arg-Trp-Gly-Lys(Msoc)-Pro-Pra-NH$_2$·HCl (**6a**). A soln. of Boc-Ser-Tyr-Ser-Met-Glu(O*t*Bu)-His-Phe-Arg-Trp-Gly-OH [14] (290 mg, 0.2 mmol), **5a** (110 mg, 0.21 mmol), and HOBt (54 mg, 0.4 mmol) in dry DMF (6 ml) was treated with DCC (52 mg, 0.25 mmol) and kept at 22° for 48 h. The DCU was filtered off and the clear soln. chromatographed over *Sephadex LH-20* (elution with DMF/H_2O 4:1). Precipitation from DMF/Et_2O yielded 322 mg (82%) of pure **6a**. TLC: R_f 0.68 (BPAW1), 0.66 (BPAW2). $[\alpha]_D^{20} = -30.0°$ ($c = 0.2$, AcOH). UV (1N AcOH): 279 (6415). Amino-acid analysis (molar ratios relative to Gly; Pra not determined): Ser 1.72, Tyr 1.21, Met 0.71, Glu 0.98, His 0.89, Phe 1.03, Arg 1.25, Trp 1.12, Gly 1.00 (R), Lys 0.89, Pro 1.22. $C_{88}H_{126}ClN_{21}O_{24}S_2$ (1961.70).

Boc-Ser-Tyr-Ser-Met-Glu(OtBu)-His-Phe-Arg-Trp-Gly-Lys(Msoc)-Pro-Nva-NH$_2$·HCl (**6b**) was prepared as described for **6a** in 78% yield. TLC: R_f 0.70 (BPAW1), 0.68 (BPAW2). $[\alpha]_D^{25} = -35.0°$ ($c = 0.2$, AcOH). UV (1N AcOH): 279 (6948). Amino-acid analysis: Ser 1.65, Tyr 1.07, Met 0.72, Glu 0.86, His 0.79, Phe 0.87, Arg 1.12, Trp 0.82, Gly 1.00 (R), Lys 0.90, Pro 1.05, Nva 0.74. $C_{88}H_{130}ClN_{21}O_{24}S_2$ (1965.73).

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys(Msoc)-Pro-Pra-NH$_2$·2HCl (**7a**). After treating **6a** (196 mg, 0.1 mmol), with 0.12N HCl in HCOOH (4 ml) for 12 min, the soln. was evaporated and traces of acid removed by repetitive evaporation of MeOH/H_2O 1:1. Precipitation from DMF/Et_2O yielded 175 mg (95%) of **7a**. TLC: R_f 0.38 (BPAW1), 0.36 (BPAW2). $C_{79}H_{111}Cl_2N_{21}O_{22}S_2$ (1841.93).

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys(Msoc)-Pro-Nva-NH$_2$·2HCl (**7b**) was prepared as described for **7a**. Yield 94%. TLC: R_f 0.40 (BPAW1), 0.38 (BPAW2). $C_{79}H_{115}Cl_2N_{21}O_{22}S_2$ (1845.96).

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Pra-NH_2·2AcOH (**8a**). The soln. of **7a** (184 mg, 0.1 mmol) in DMF/pyridine 3:1 (3 ml) was treated with 4-nitrophenyl acetate (27 mg, 0.15 mmol) and *N*-ethylmorpholine (11.5 mg, 0.1 mmol). After 36 h at 22°, the soln. was filtered through *Sephadex LH-20* (elution with DMF/H_2O 4:1), and the homogeneous fractions were concentrated and precipitated with Et_2O. The dry powder of protected **7a** (166 mg, 0.09 mmol) was dissolved in DMF/MeOH 1:1 (4 ml) and treated with 0.1N NaOH (4 ml) for 5 min at 20°. The reaction was stopped by addition of 0.1N HCl at 0°, and the soln. was concentrated *in vacuo*. The residue was dissolved in NH_4OAc (0.01M, pH 6) and chromatographed on carboxymethyl-cellulose (*Whatman, CM52*) with a linear gradient of NH_4OAc (0.05M to 0.5M, pH 6). The homogeneous fractions were filtered through *Sephadex LH-20* (elution with 1% aq. AcOH) and lyophylized. Yield 109 mg (61%). TLC: R_f 0.34 (BPAW1), 0.32 (BPAW2). TLE (R(Arg)): 0.56 (pH 5.6), 0.62 (pH 3.6). HPLC: t_R 18.7 min (one single peak). $[\alpha]_D^{25} = -58.5°$ ($c = 0.33$, 1% aq. AcOH). UV (0.1N NaOH): 281 (6830), 289 (6520); Tyr/Trp = 0.95. Amino-acid analysis (Pra not determined): Ser 1.75, Tyr 1.20, Met 0.88, Glu 1.02, His 0.86, Phe 1.04, Arg 0.93, Trp 0.75, Gly 1.00 (R), Lys 0.94, Pro 1.26. $C_{81}H_{113}N_{21}O_{23}S$ (1780.99).

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Nva-NH_2·2AcOH (**8b**) was prepared as described for **8a**, except that the final product was purified by repetitive (2 ×) filtration through *Sephadex LH-20* (elution with 1% aq. AcOH). Yield 75%. TLC: R_f 0.35 (BPAW1), 0.33 (BPAW2). TLE (R(Arg)): 0.56 (pH 5.6), 0.62 (pH 3.6). HPLC: t_R 19.2 min (one single peak). $[\alpha]_D^{25} = -60.3°$ ($c = 0.33$, 1% aq. AcOH). UV (0.1N NaOH): 281 (6815), 289 (6505); Tyr/Trp = 0.92. Amino-acid analysis: Ser 1.69, Tyr 1.05, Met 0.85, Glu 1.15, His 0.91, Phe 1.08, Arg 0.91, Trp 0.72, Gly 1.00 (R), Lys 0.94, Pro 1.22, Nva 0.94. $C_{81}H_{117}N_{21}O_{23}S$ (1785.02).

In another experiment, **8a** (18 mg, 10 μmol) was dissolved in DMF (4 ml) containing 5% HMPTA and was hydrogenated for 15 min in the presence of 10% Pd/C (12 mg). The catalyst was removed by centrifugation, the solvent evaporated, and the peptide filtered through *Sephadex LH-20* and lyophilized from dil. AcOH. Yield 12 mg (66%). HPLC analysis proved the identity of this product with **8b**.

Tritiations. – *General.* The peptides were dissolved in an anh. solvent (DMF or DMF/HMPTA). The soln. was placed into a 50-ml flask (RV) together with a magnetic stirrer. The catalyst which had been dried under high vacuum overnight was kept in a small metal container held above the soln. with a magnet. The RV was attached to the $^3H_2$ apparatus, and the soln. was frozen in liq. N_2. The system was evacuated to $< 10^{-3}$ mbar, the soln. was degassed by repetitive thawing and freezing whereby the system was re-evacuated. After the pressure had reached a constant value of $< 10^{-3}$ mbar, valves V5, V3, and V15 were closed and V8/V8′ opened. The unit (in between V3-V9-V10-V11-V12-V13-V14-V15-V16) was now filled with $^3H_2$ gas by heating UT1 until the differential pressure gauge indicated 1250 Torr (1650 mbar). V8′ was closed and V1 opened whereby the pressure decreased. More $^3H_2$ was added in the same way until the unit, including RV, had reached the maximal pressure. V8/V8′ and V4 were closed, and the reaction was started by thawing the soln. in the RV and pouring the catalyst into the soln. After vigorous stirring at r.t. for 15 min, the soln. was frozen in liq. N_2 and V8/V8′ opened in order to adsorb the $^3H_2$ gas of the unit onto UT1. From the pressure difference between start and end of the reaction, the incorporation of ^3H into the peptide and the solvent could be calculated: 1 Torr corresponded to about 0.25 Ci of $^3H_2$ gas. Excess $^3H_2$ dissolved in the reaction mixture was eliminated by repetitive thawing and freezing and simultanous pumping through the FUT with the aid of the *Toepler* pump; the FUT was used for this purpose because traces of solvent, He, or I_2 would contaminate the other uranium traps and hence inhibit the readsorption reaction of $^3H_2$. The unit was heated with a welding-torch when it was still evacuated in order to get rid of any solvent traces left in the system. The solvent trap (ST) was now attached to the unit, immersed in liq. N_2 and the solvent distilled off by gently heating the RV. New solvent (EtOH/H_2O) was added 2 or 3 times and distilled off in order to exchange loosely bound ^3H. The almost dry residue was treated with 10 ml of DMF/H_2O 1:1 and then distributed into 12 *Eppendorf* tubes and centrifuged inside the hood in order to remove the catalyst. The clear soln. was pipetted off into a round-bottom flask and the solvent evaporated in a *Büchi Rotavapor-M* also installed inside the hood. After several evaporations from DMF/H_2O, most of the non-covalently incorporated ^3H-atoms had been removed, and the peptide soln. was now ready for transport in dry ice (sealed as usual in a tin).

H-Lys(Msoc)-Pro-Nva($^3H_4$)-NH_2·HCl (**9**). A soln. of **5a** (10.5 mg, 20 μmol) in anh. DMF (6 ml) was treated with $^3H_2$ gas at 1640 mbar in the presence of 10% Pd/C (20 mg; *Fluka*). The peptide was processed as described above, and after precipitation from MeOH/Et_2O, 6.8 mg (65%) of pure **9** were obtained. TLC: R_f 0.45 (BPAW1; identical with **5b**). Specific activity of **9**: 116 Ci/mmol ± 8%.

Boc-Ser-Tyr-Ser-Met-Glu(OtBu)-His-Phe-Arg-Trp-Gly-Lys(Msoc)-Pro-Nva($^3H_4$)-NH_2·HCl (**10**). A soln. of Boc-Ser-Tyr-Ser-Met-Glu(O*t*Bu)-His-Phe-Arg-Trp-Gly-OH [14] (4.4 mg, 3 μmol), **9** (1.6 mg, 3 μmol), and HOBt (1.2 mg, 9 μmol) in DMF (500 μl) was treated with DCC (2 mg, 9 μmol) and kept at 20° for 16 h. The peptide was purified as described for **6a**: 4.6 mg (78%) of homogenous **10**. TLC: R_f 0.68 (BPAW1).

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys(Msoc)-Pro-Nva($^3H_4$)-NH_2·2HCl (**11**). A soln. of **10** (4.6 mg, 2.3 μmol) in 0.12N HCl in HCOOH (1 ml) was kept at 20° for 12 min and evaporated as described for **7a**: 4.1 mg (94%). TLC: R_f 0.40 (BPAW1).

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Nva($^3H_4$)-NH_2·3AcOH (**12**). A soln. of **11** (2.1 mg, 1.1 μmol) in DMF/MeOH/0.1N NaOH 1:1:2 (300 μl) was kept at 20° for 5 min and then neutralized with 0.1N HCl. The residue was dissolved in 1% aq. AcOH (1 ml), passed through weakly basic ion exchanger *(Merck II)*, and then chromatographed over *Sephadex LH-20*: 1.65 mg (81%; determined spectroscopically). TLC: R_f 0.32 (BPAW1). TLE (R(Arg)): 0.66 (pH 5.6). HPLC: t_R 17.4 min (one major peak with a tiny trace of oxidized material at 15.9). Specific activity: 115 Ci/mmol ± 6%. Amino-acid analysis: Ser 1.63, Tyr 1.13, Met 0.79, Glu 0.95, His 0.85, Phe 1.08, Arg 1.14, Trp 0.93, Gly 1.00 (R), Lys 0.84, Pro 1.22, Nva 0.88.

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Nva($^3H_4$)-NH_2·2AcOH (**13**). a) A soln. of **11** (1.8 mg, 1 μmol) in DMF/pyridine 3:1 (200 μl) was treated with 4-nitrophenyl acetate (0.27 mg, 1.5 μmol) and *N*-ethylmorpholine (0.12 mg, 1 μmol) for 16 h. The Msoc group was then split off as described for **12** and the peptide purified as outlined below: 1.1 mg (61%) of **13I**; overall-yield from **9** to **13I**: 29%. TLC: R_f 0.35 (BPAW1). TLE (R(Arg)): 0.56 (pH 5.6). HPLC: t_R 19.2 min (identical with **8b** and **13II**). Specific activity: 115 Ci/mmol ± 7%.

b) A soln. of **8a** (8.9 mg, 5 μmol) in anh. DMF/HMPTA 99:1 (6 ml) was treated with $^3H_2$ gas at 1645 mbar in the presence of 10% Pd/C (15 mg). The peptide was passed through weakly basic ion exchanger *(Merck II)* and purified by filtration through *Sephadex LH-20* (elution with 1% aq. AcOH): 2.9 mg (35%; based on amino-acid analysis) of **13II**. TLC: R_f 0.35 (BPAW1). TLE (R(Arg)): 0.56 (pH 5.6). HPLC: t_R 19.2 min (one major peak, identical with **8b**, with a tiny peak of oxidized material at 17.6 min; see *Fig. 3*). Specific activity: 115 Ci/mmol ± 5%. UV (1% aq. AcOH): 281 (6880). Amino-acid analysis: Ser 1.70, Tyr 1.10, Met 0.92, Glu 0.95, His 1.15, Phe 0.90, Arg 0.98, Trp 0.90, Gly 1.00 (R), Lys 0.85, Pro 1.20, Nva 0.80.

Chymotryptic Hydrolysis of **13I/II** was performed as described in [2]. The tryptic fragments were separated in BPAW2 and the relative ^3H content determined: R_f 0.14 *(H-Gly-Lys-Pro-Nva-NH_2)*, 99.5% (**13I**)/93% (**13II**); R_f 0.24 *(H-Ser-Met-Glu-His-Phe-OH)*, 0.1% (**13I**)/5% (**13II**); R_f 0.34 *(H-Arg-Trp-OH)*, 0.1% (**13I**)/1% (**13II**); R_f 0.43 *(Ac-Ser-Tyr-OH)*, 0.1% (**13I**)/1% (**13II**).

H-Ser-Tyr($^3H_2$)-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-($^3H_2$)-Pro-OH·8AcOH (**14**). A soln. of protected [Tyr(I_2)^{2,23}]ACTH(1–24) [4] (5 mg) in anh. DMF (5 ml) was exposed to $^3H_2$ gas in the presence of 6 mg each of 5% Pd/C and 5% Rh/$CaCO_3$. The product was then treated with CF_3COOH (3 ml) containing 3% of anisole and 7% of H_2O at 22° for 30 min. The residue was passed through weakly basic ion exchanger *(Merck II)* and purified by chromatography on *Sephadex LH-20*. Yield 45%. HPLC: t_R 23.5 min. Specific activity: 100 Ci/mmol ± 10%. Amino-acid analysis: Ser 1.71, Tyr 2.21, Met 0.82, Glu 1.12, His 0.92, Phe 1.08, Arg 2.73, Trp 0.91, Gly 2.00 (R), Lys 3.64, Pro 3.33, Val 2.89.

Storage of Peptides. The peptides were diluted to a final concentration of 10^{-5}M (*ca.* 1 mCi/ml) and 10^{-6}M (*ca.* 0.1 mCi/ml) with 1mM HCl containing 0.1% of bovine serum albumin and 5% of EtOH, distributed into 1-ml vials, and stored at − 70° or −196°.

Bioassays. – *Pigment-dispersing activities* of **8a**, **8b**, **12**, **13**, α-MSH, and des-acetyl-α-MSH were determined with the modified [9] reflectometric test system of *Shizume et al.* [22] using isolated skin of the leopard frog *Rana pipiens* and with a mixed reflectometric/visual lizard melanophore assay [18] using isolated skin of the American chamaeleon *Anolis carolinensis*.

Tyrosinase-stimulating activities of the peptides were measured with *Cloudman S-91* mouse melanoma cells in tissue culture using 24-well *Costar* dishes with 5×10^4 cells/well which were incubated in 2 ml of *Ham's F-10* medium containing 5% of inactivated horse serum, 0.2 μCi [3′,5′,-$^3H_2$]-L-tyrosine, 0.04 μg of isobutylmethylxanthine, and the peptides in the appropriate dilution. After 48 h, [^{3}H]tyrosine was separated from ^{3}HHO by treatment of 1 ml of medium with 40 mg of charcoal and centrifugation, and the supernatant was counted in the beta-counter.

Steroidogenesis of **14** was determined with a rat adrenocortical cell assay [17]: dispersed *fasciculata/reticularis* cells, 10^5 cells/ml, were preincubated in *Krebs-Ringer* hydrogen carbonate/glucose buffer containing 5.9 mM K^+ and 0.5% of human serum albumin for 1 h. The cells were then incubated in the presence of various concentrations of **14** and of ACTH(1–24) *(Synacthen)* for 1 further h. Corticosterone in the medium was determined by specific radioimmunoassay without extraction.

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