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IODINATION ASSOCIATED INACTIVATION OF β-MELANOCYTE STIMULATING HORMONE

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SUMMARY: Contrary to other published reports, iodinated β-melanocyte stimulating hormone (β-MSH) is without biological activity as measured by frog skin bioassay, melanoma (mouse S-91) adenylate cyclase assay, or melanoma tyrosinase assay. Inactivation results in part from oxidation of the methionine residue by the chloramine T and sodium metabisulfite used in the iodination reaction. Replacement of the methionine of β-MSH with norleucine by solid phase synthesis results in an analogue which is more resistant, but not completely resistant, to inactivation. Thus, in order to obtain a biologically active radioligand for radioreceptor studies, further tailoring of the hormone and/or modification of the iodination procedure will be needed.

Melanocyte stimulating hormone (MSH) interaction with melanoma cells results in activation of adenylate cyclase (1) with resulting elevation of intracellular cyclic AMP levels (2). Utilizing techniques developed for other peptide hormones, radioiodinated MSH has been used to study receptor binding of the hormone to melanoma cells (3). The preparation of a biologically active iodinated β-MSH which purportedly interacts with melanoma cell receptors at a specific stage of the cell cycle has been reported (4). In contrast, we demonstrate here that iodination of β-MSH under the conditions reported by these workers leads to complete loss of the biological activity of the hormone as demonstrated by frog skin bioassay, melanoma adenylate cyclase assay, and melanoma tyrosinase assay.

MATERIALS AND METHODS

Materials: ATP, GTP, cAMP, creatine phosphate, creatine phosphokinase, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), and bovine serum albumin were obtained from Sigma Chemical Co. 1-Methyl-3-isobutylxanthine was obtained from Aldrich Chemical Company. [^{3}H]cyclic AMP (specific activity 34.3 Ci/mmol), [α-^{32}P]ATP (specific activity 20-30 Ci/mmol) and [^{3}H]tyrosine (specific activity 48 Ci/mmol) were purchased from New England Nuclear.

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Peptide Synthesis: β-MSH and [7-norleucine]β-MSH were synthesized using a chloromethylated polystyrene resin cross-linked with 1% divinylbenzine. The C-terminal amino acid was attached to the resin at its benzyl ester by the Merrifield procedure (5). Details of our synthetic procedure closely parallel our previous methods (6) and will be reported elsewhere.

Iodination: Iodination of both peptides was done according to the method of Hunter and Greenwood (7) as modified by Varga et al. (3). The reactions were carried out using ^{127}I (not radioactive) on a larger scale than reported but maintaining the same relative concentrations and molar ratios of the reactants as follows: 0.25 mg (10^{-7} mole) of each peptide was treated for exactly 15 sec at room temperature with 2.27 x 10^{-7} mole of NaI and/or 2.59 x 10^{-5} mole of chloramine T (CT) in a total reaction volume of 5 ml of phosphate buffered saline (PBS 0.1 M, pH 7.4). The reaction was stopped by the addition of 10 ml of a 2.4 g/l solution of sodium metabisulfite in PBS. All samples were then diluted with distilled water and frozen at -20°C until ready to be assayed.

Cell Cultures: Cloudman S-91 3960 (CCL 53.1) melanoma cells were obtained from the American Type Culture Collection Cell respository. Cells were grown in Ham's F-10 medium fortified with 2% fetal calf serum and 10% horse serum. Penicillin/streptomycin (100 units/ml, 100 ug/ml, respectively) was also present in the medium. Stock cultures were subcultured weekly. For experiments, cell were seeded into 25 cm^2 Corning flasks at a density of 5 X 10^5 cells/flask and were allowed to attach overnight. Cell counts were made using a hemocytometer.

Tyrosinase Assays: At the start of experiments the medium was removed from the cells and replaced with fresh medium containing 1 uCi/ml [^3H]tyrosine (specific activity, 48 Ci/mmole) and the hormonal or other agents under investigation. The medium removed from the cells at various times was assayed for the presence of ^3H_2O using a modification of the charcoal absorption method of Pomerantz (8). Following absorption of [^3H]tyrosine by charcoal, 1 ml of the liquid phase was passed over a Dowex 50W column (Bio-Rad Laboratories) equilibrated with 0.1 M citric acid. This step removed residual [^3H]tyrosine. The eluent from the Dowex column was collected directly into scintillation vials and counted in a Beckman LS-8000 scintillation spectrometer.

Melanoma Membrane Preparation: Melanotic tumor-bearing mice (DBA/2J) were sacrificed about 4 to 5 wks following subcutaneous injection of 2-4 X 10^6 cells. The tumors frequently measured 2 to 3 cm in diameter. Encapsulated melanoma tumors were excised, dissected from contaminating tissue and transferred immediately to 1 mM $NaHCO_3$ buffer, pH 7.5, maintained at 0°C. All of the following manipulations were conducted at 0°C. Minces of melanoma tumor were homogenized via 8 strokes in a loose-fitting Dounce homogenizer (type B pestle, Wheaton), stirred for 3 min and filtered through 4 layers of cheesecloth. The filtrate was further homogenized with 24 strokes in a Wheaton homogenizer with B pestle, followed by 8 strokes with a type A pestle. The resultant crude homogenate was centrifuged for 10 min at 10,000 X g. The pellet was resuspended in fresh 1 mM $NaHCO_3$ buffer and centrifuged for 10 min at 10,000 X g to obtain a washed membrane particulate fraction.

Adenylate Cyclase Assay: Adenylate cyclase activity was assayed in a medium containing 1 mM (α-^{32}P]ATP (specific activity 30 to 60 cpm/pmol), 5 mM $MgCl_2$, 1 mM cAMP, 1 mM MIX, 1mM DTT, 20 mM creatine phosphate, 100 U/ml creatine phosphokinase and 10 μM GTP in 30 mM Tris·HCl, pH 7.5. Reactions were initiated by the addition of 40 μl of membrane fraction (100 μg of protein) to 60 μl of reaction mixture. After incubation at 30°C for 10 min the reactions were terminated by the addition of 100 μl of 2% SDS containing 5 mM ATP and 1.4 mM [^3H]cAMP (approximately 0.01 μCi, to measure recovery) at pH 7.5. This was immediately followed by heating for 3 min at 100°C. The

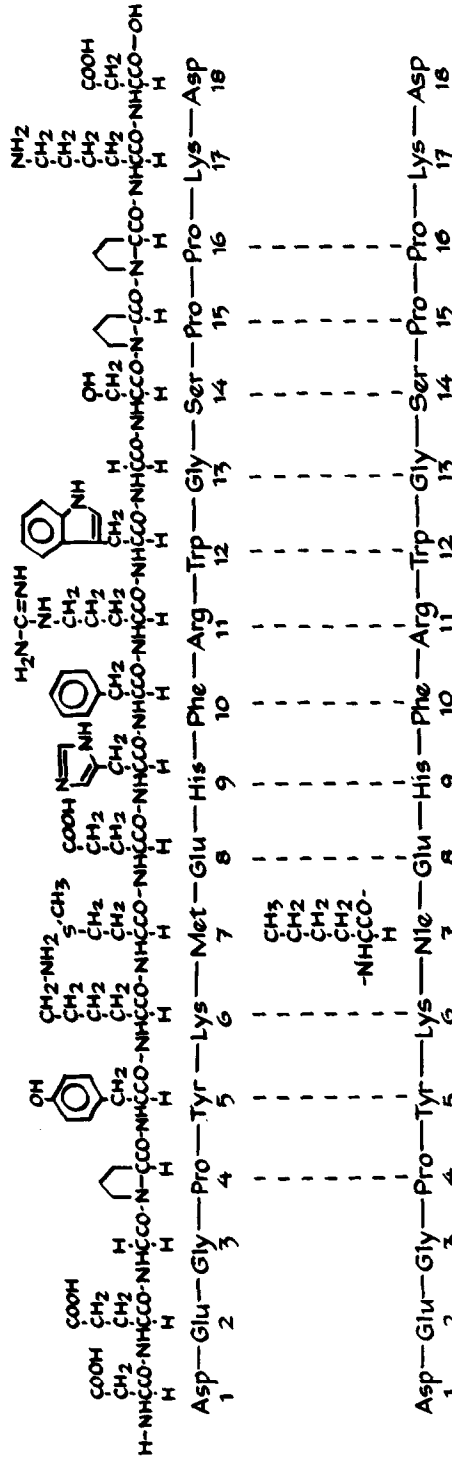

Figure 1: Comparison of the structural formulas of β-MSH (upper peptide) and [Nle^7]β-MSH (lower peptide).

purification of [^{32}P]cAMP was carried out by the method of Salomon et al. (9). Specific adenylate cyclase activities are expressed as picomoles of cAMP formed per 10 min per mg of protein.

Frog Skin Bioassay: β-MSH and [norleucine^7]β-MSH ([Nle^7]β-MSH) were bioassayed as described previously (10). This assay measures the effects of MSH and other agents on amphibian integumental chromatophores (11). The animals used in this study were Rana pipiens obtained from Nasco, Fort Atkinson, Wisconsin.

RESULTS AND DISCUSSION

β-MSH (Figure 1) was iodinated by methods identical to those published by Varga et al. (3). In contrast to their reported results, we found that β-MSH was completely inactivated as determined by the frog skin bioassay (Table 1). Not only was the activity abolished by iodination, but chloramine T (CT) and sodium metabisulfite ($Na_2S_2O_5$), the reactants used in the iodination procedure, each inactivated the hormone (Table 1). Similar results were obtained upon iodination of α-MSH (12).

It has been suggested that CT inactivates adrenocorticotropin (13) by oxidation of the methionyl residue near the so-called "active site" of the molecule. To try to obviate this oxidation problem we synthesized [Nle^7]β-MSH with norleucine substituted for the methionine at the 7 position of the peptide (Figure 1). This analogue was also inactivated by the iodination procedure and by the individual action of the CT. $Na_2S_2O_5$, however, had no significant effect on this analogue (Table 1). These data differ from our results with the corresponding norleucine analog of α-MSH which is resistant to chloramine T (and $Na_2S_2O_5$) inactivation (12).

Varga et al. (3) reported that iodinated β-MSH retained its original biological activity and then used it to study MSH receptors on melanoma cells. Therefore, we decided to utilize melanoma cells to determine the biological activities of our iodinated peptides. As anticipated, β-MSH and [Nle^7]β-MSH greatly increased the activities of both adenylate cyclase (Table II) and tyrosinase (Table III). However, upon iodination the ability of the peptides to stimulate these enzymes was completely abolished (Tables II & III). [Nle^7]β-MSH was apparently more resistant than β-MSH itself to oxidation by CT +

Table I. Effects of Iodination and Iodination Reactants on the Biological Activity of β-MSH and [Nle^7] β-MSH as Determined In Vitro by the Frog Skin Bioassay

Sample--From Iodination Reactions (Peptide Concentration = 10^{-8}M)	% Response* (frog skin darkening)	P**
Control	3.7 ± 1.1	
Chloramine T (CT) + NaI + $Na_2S_2O_5$	7.4 ± 1.5	NS
β-MSH	57.4 ± 6.7	< 0.002
β-MSH + CT	7.0 ± 1.8	NS
β-MSH + $Na_2S_2O_5$	4.2 ± 2.3	NS
β-MSH + NaI	54.4 ± 2.9	< 0.001
β-MSH + CT + $Na_2S_2O_5$	19.8 ± 5.4	NS (<0.10)
β-MSH + CT + NaI	2.7 ± 1.1	NS
β-MSH + CT + NaI + $Na_2S_2O_5$	2.6 ± 2.8	NS
Control	3.7 ± 1.1	
Chloramine T (CT) + NaI + $Na_2S_2O_5$	10.0 ± 3.5	NS
[Nle^7] β-MSH	59.4 ± 6.7	< 0.001
[Nle^7] β-MSH + CT	32.7 ± 2.7	< 0.001
[Nle^7]β-MSH + $Na_2S_2O_5$	62.5 ± 6.9	< 0.001
[Nle^7]β-MSH + NaI	65.0 ± 5.4	< 0.001
[Nle^7]β-MSH + CT + $Na_2S_2O_5$	62.2 ± 5.8	< 0.001
[Nle^7]β-MSH + CT + NaI	6.9 ± 1.2	NS
[Nle^7]β-MSH + CT + NaI + $Na_2S_2O_5$	32.8 ± 6.5	< 0.01

*Each value represents the mean, ± S.E., percent response of the skins (3 per group in the first experiment and 5 per group in the second experiment) under each experimental condition.

**Significant or nonsignificant (NS) differences (Student's t-test) between the control and experimental groups are indicated.

$Na_2S_2O_5$. These data are basically consistant with those obtained using the frog skin bioassay.

Our results, therefore, demonstrate that the iodination procedures utilized in these studies destroy the biological activity of β-MSH whether studied on frog skin melanocytes or melanoma cells. The data obviously do not support the claim of Varga et al., (3) that iodinated β-MSH retains full

Table II. Effects of Iodination and Iodination Reactants on β-MSH and [Nle^7]β-MSH Stimulated Melanoma Adenylate Cyclase Activity

Sample--From Iodination Reactions (Peptide Concentration = 6 X 10-^{6}M)	Specific Activities* (pmol cAMP/10 min/mg protein)	P**
Control	16.4 ± 1.0	
Chloramine T (CT) + NaI + $Na_2S_2O_5$	18.5 ± 3.2	NS
β-MSH	48.2 ± 1.1	< 0.001
β-MSH + CT + $Na_2S_2O_5$	17.3 ± 1.0	NS
β-MSH + CT + NaI + $Na_2S_2O_5$	16.8 ± 1.2	NS
[Nle^7]β-MSH	85.1 ± 1.0	< 0.001
[Nle^7]β-MSH + CT + $Na_2S_2O_5$	21.2 ± 1.0	NS
[Nle^7]β-MSH + CT + NaI + $Na_2S_2O_5$	17.6 ± 2.2	NS

*Results represent the means of three determinations ± S.E.

**Significant or nonsignificant (NS) differences (Student's t-test) between the control and the experimental groups are indicated.

biological activity. Rather they suggest that a number of independent processes are involved in the inactivation of β-MSH. Chloramine T as well as $Na_2S_2O_5$ oxidation of the methionyl residue and possibly other sites in the β-MSH molecule results in inactivation of the hormone. In addition, it is quite possible, as indicated in our studies on α-MSH, that iodine incorporation into the peptide also reduces its biological activity (12).

The discrepancies between our results and those of Varga et al. (3) are difficult to explain. In other iodination studies using Na^{125}I (unpublished data), incorporation of label into the molecule was readily achieved, however, the specific radioactivity of our product never exceeded 1000 Ci/mmol (as opposed to the 2000 Ci/mmole reported by Varga et al. (3). In addition, iodinations resulting in specific activities greater than 200 Ci/mmol consistantly resulted in loss of biological activity of the peptide. We, therefore, suggest that iodination of MSH to high specific radioactivity (2000 Ci/mmol) using the chloramine T oxidation technique described by Varga et al. (3) re-

Table III. Effects of Iodination and Iodination Reactants on β-MSH and [Nle^7] β-MSH Stimulated Melanoma Tyrosinase Activity

Sample--From Iodination Reactions (Peptide Concentration = 6.5 X 10^{-8}M)	Tyrosinase Activity* (% of control)
Control	100 ± 2.0
Chloramine T (CT) + NaI + $Na_2S_2O_5$	127 ± 2.8
β-MSH	723 ± 3.2
β-MSH + (CT + NaI + $Na_2S_2O_5$ added directly to cell culture)	701 ± 1.4
β-MSH + CT + $Na_2S_2O_5$	138 ± 4.4
β-MSH + NaI + CT + $Na_2S_2O_5$	113 ± 3.6
[Nle^7] β-MSH	769 ± 1.3
[Nle^7] β-MSH + (CT + NaI + $Na_2S_2O_5$ added directly to cell culture)	767 ± 3.0
[Nle^7] β-MSH + CT + $Na_2S_2O_5$	382 ± 5.1
[Nle^7] β-MSH + NaI + CT + $Na_2S_2O_5$	135 ± 6.4

*Melanoma cells (5 X 10^{-5}) were seeded into 25 cm^2 culture flasks and allowed to attach overnight. At the start of the experiment medium was replaced with 4 ml of medium containing 1 uCi/ml of [^{3}H]tyrosine and the compounds under investigation. Medium was changed every 24 hr and assayed for ^3H_2O as described in MATERIALS AND METHODS. Data from 48 hr samples are shown. Values are the averages of 4 determinations ± S.D.

sults in a biologically inactive product. Therefore, we feel that a reevaluation of any data (3,4) obtained using ^{125}I-labeled MSH prepared by this method is in order.

It is obvious that an iodinated MSH or related analogue of high specific radioactivity would be extremely useful in a radioreceptor assay. Not only would such a peptide be important for clarifying receptor parameters related to melanoma cell activation but it would facilitate studies on other tissues that might possess such receptors. It is clear from the present studies that such a molecule is not yet available. Our data suggest that iodinated β-MSH prepared by the method of Varga et al. (3) cannot be utilized as the

radioligand in a radioreceptor assay for MSH. However, our results with α-MSH suggest that this shorter peptide, or rather an analogue thereof, might eventually be utilized as a radioligand in such studies (12).

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