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Research Article

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Radioimmunoassay of β-MSH in Human Plasma and Tissues *

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Abstract. A radioimmunoassay method for β-melanocyte-stimulating hormone (β-MSH) has been developed and utilized in the identification and quantification of this hormone in human plasma and tissues. The concentration of β-MSH in two human pituitary glands was found to be approximately 350 μg/g. β-MSH was identified in the tumor tissue of all 11 patients with the ectopic ACTH syndrome who were studied; concentrations in indivdual cases ranged from 3 to 1600 ng/g. In plasma of chronically hyperpigmented patients with Addison's disease, Cushing's disease (after bilateral adrenalectomy), and the ectopic ACTH syndrome, β-MSH concentrations of 0.5–6 ng/ml were found. The degree of clinical hyperpigmentation was well correlated with the quantity of β-MSH in the plasma. β-MSH concentrations in the plasma of normal subjects were less than 0.09 ng/ml. In all of these circumstances, bioassays for MSH were also performed, and it was found that most of the biologic MSH activity of the plasma and tissues could be accounted for by β-MSH.

Introduction

It is well known that hyperpigmentation occurs in Addison's disease (1), in some patients who have undergone adrenalectomy for Cushing's disease (2), and in some patients with the ectopic ACTH syndrome (3–6). Previous studies in our laboratory have shown that the principal melanocyte-stimulating factor in the plasma of such patients is different from ACTH (7, 8) but have failed to identify it more specifically. In a recent study (9), we identified α-melanocyte-stimulating hormone (α-MSH) in human pituitaries and tumors by means of radioimmunoassay, but this substance accounted for only a small percentage of the total melanocyte-stimulating activity of these tissues as determined by bioassay.

We therefore postulated that the major melanocyte-stimulating factor in human tissues and plasma might be β-MSH. This point had already been established for pituitary tissue (10) but remained unexplored with respect to plasma or nonpituitary tumors that produce "ectopic" ACTH and MSH. In order to examine this question, it was necessary to develop a sensitive and specific radioimmunoassay for β-MSH. The results of the radioimmunoassays were compared with those of bioassays in order to determine what proportion of the total melanocyte-stimulating activity of a given specimen could be attributed to its content of β-MSH.

Methods

β-MSH antisera were obtained by immunizing guinea pigs with the β-MSH contained in commercial ACTH.[1]

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[1] Corticotrophin-Zinc 40 U/ml, Organon Inc., West Orange, N. J.

Four different treatment schedules, each lasting for about 2 months, were employed. Some guinea pigs were injected intramuscularly with unaltered commercial ACTH in doses in 40 U once a week. Some were similarly injected in doses of 20 U twice a week. Another group of guinea pigs received weekly subcutaneous injections containing 20 U of the commercial ACTH emulsified with Freund's complete adjuvant. The fourth group received subcutaneous injections of commercial ACTH in Freund's complete adjuvant in doses of 40 U every 2 wk. 10 days after the last injection, blood was drawn by cardiac puncture and plasma was preserved as previously described (9). Several guinea pigs in each group were found to have developed antibodies to β-MSH. After a respite of 2 months, immunization of these guinea pigs was resumed. Five weekly intramuscular injections of 40 U of commercial ACTH alone were given to those animals previously immunized with commercial ACTH only. Five weekly subcutaneous injections of 20 U of commercial ACTH emulsified with complete Freund's adjuvant were given to those guinea pigs previously injected with this material. 10 days after the last of these injections, blood was again drawn and plasma assayed for antibodies to β-MSH.

In preparation for radioimmunoassay, synthetic human β-MSH (β_h-MSH) was labeled [2] with ^{131}I by the method of Hunter and Greenwood (11) and was then purified on a cellulose column (12) and used within 1 day to avoid accumulation of "damaged" β_h-MSH–^{131}I during storage and preserve the high specific activity of the labeled hormone. The labeled β-MSH employed in radioimmunoassays had specific activities of 0.5–1.0 c/mg of β-MSH.

The binding of β-MSH–^{131}I to antibodies and its displacement by unlabeled β-MSH were studied by the chromatoelectrophoretic system of Yalow and Berson (13). The standard diluent for the incubation mixture consisted of 100 ml of 0.075 M phosphate buffer (pH 7.2) containing 0.45 g of NaCl, 0.25 g of human serum albumin, 1 ml of normal guinea pig serum and 0.01 g of Merthiolate. Acidified human albumin solution (9) was used as a vehicle for β-MSH, other polypeptides, and biologic extracts.

Incubation mixtures were prepared by mixing 0.13 ml of standard diluent with 0.02 ml of standard β_h-MSH or biological extract (in acidified human albumin), 0.05 ml

[2] Na-^{131}I 350–600 mc/ml, carrier free, was obtained from Isoserve Inc., Division of Cambridge Nuclear Corporation, Cambridge, Mass.

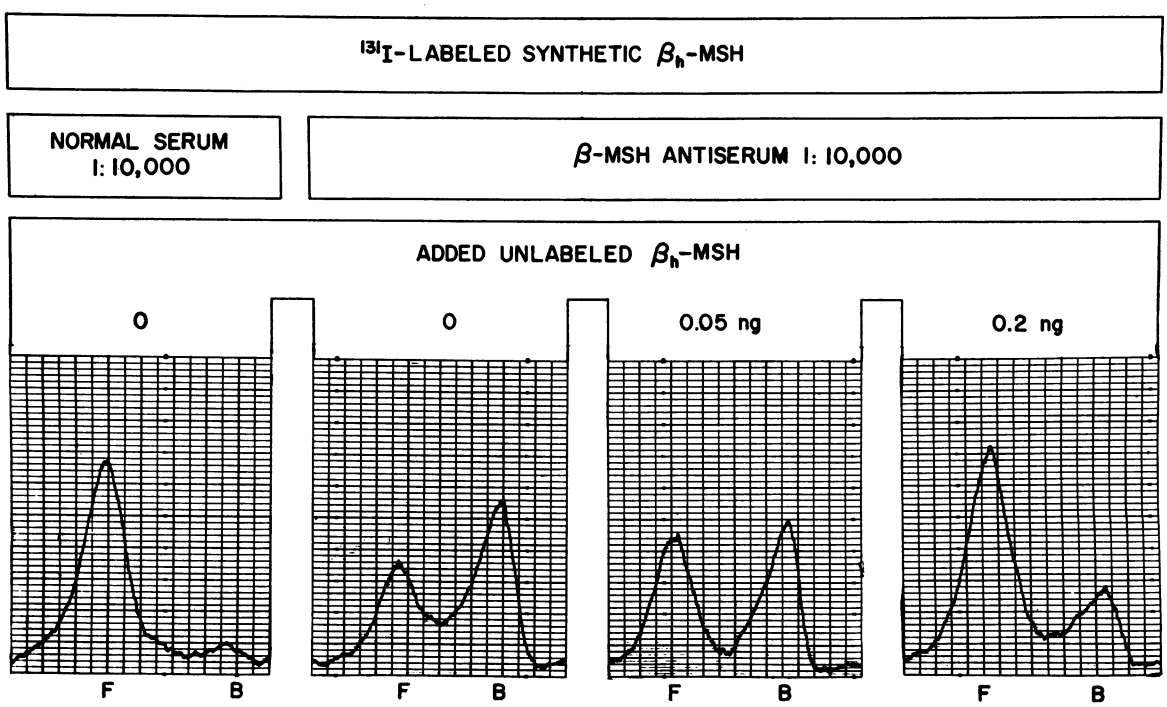

FIG. 1. CHROMATOELECTROPHORETIC MOBILITY OF β_h-MSH–^{131}I. The location of labeled hormone at the completion of chromatoelectrophoresis is represented on the abscissa. The quantity of radioactivity is represented on the ordinate. The first scan shows that, when incubated with normal guinea pig serum, β_h-MSH–^{131}I was "free" (*F*) and remained at the origin. The second scan shows that, when incubated with the antiserum, most of the β_h-MSH–^{131}I was "bound" (*B*) and migrated with the globulin fraction. The third and fourth scans show that the addition of progressively greater quantities of unlabeled β_h-MSH (0.05 ng, 0.2 ng) to the mixture resulted in progressively greater displacement of radioactivity from the "bound" peak to the "free" peak and a decrease in B/F ratio.

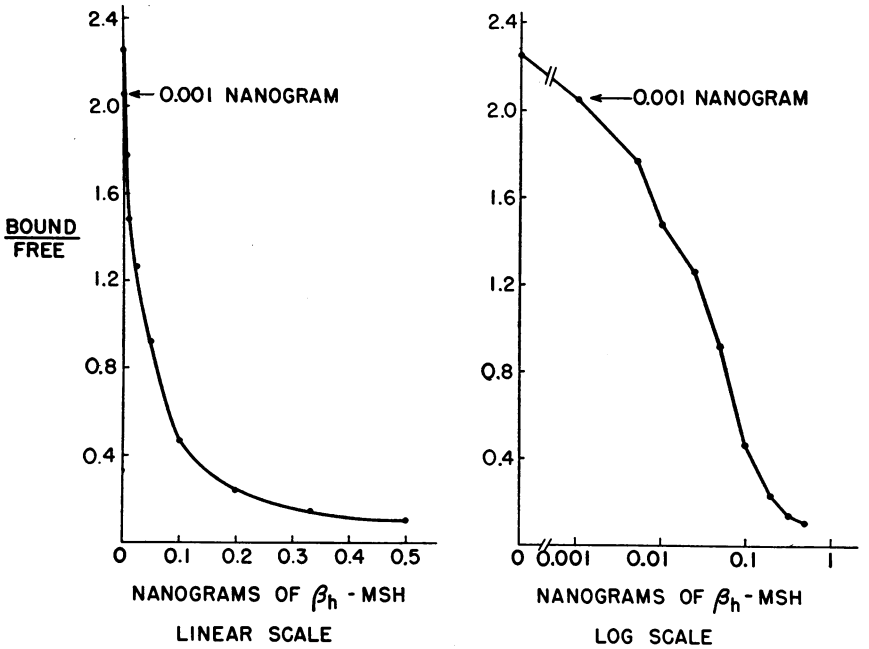

FIG. 2. STANDARD CURVE OF β-MSH RADIOIMMUNOASSAY. The ratio of the amount of bound β_h-MSH-^{131}I to the amount of free β_h-MSH-^{131}I is plotted on the ordinate. On the left, the amount of unlabeled synthetic β_h-MSH added to the incubation mixture is plotted on the abscissa using a linear scale. On the right, the same data are shown using a logarithmic scale.

of antiserum diluted appropriately with standard diluent, and β_h-MSH-^{131}I diluted with standard diluent so that 0.05 ml contained approximately 4000 dpm. Final antibody concentration was 1 : 10,000. All tubes were prepared in duplicate. Each biological extract was assayed at three or more dilutions. The amount of "damaged" β_h-MSH-^{131}I produced during incubation and chromatoelectrophoresis, although usually negligible, was routinely assessed by a method previously described (9). Incubation was carried out at 4°C for 4 days. Just before chromatoelectrophoresis 0.1 ml of normal human plasma [3] containing a trace of bromphenol blue was added to each incubation mixture to serve as a carrier. The contents of each tube were applied to a strip of Whatman 3MC paper, and the strips were subjected to chromatoelectrophoresis. The strips were dried and scanned as previously described (9). In this system unbound (or "free") β_h-MSH-^{131}I remained at the origin, whereas β_h-MSH-^{131}I bound to antibodies migrated with the globulin fraction. The areas under the "bound" peak (B) and "free" peak (F) were measured, and the ratio B/F was calculated. The B/F ratio was used as an index of the quantity of unlabeled β-MSH in a given tube (Fig. 1). Using known quantities of synthetic β_h-MSH we constructed a standard curve as a part of each experiment, and as a criterion for identifying and quantifying β-MSH in any of the unknowns it was required that their displacement curves be parallel to the dose-discriminating portion of the standard.

Samples of human pituitary tissue were extracted with glacial acetic acid, evaporated, and reconstituted in saline. The pH of the reconstituted residue was adjusted to 3 with 1% HCl. Samples of tumor from patients with the ectopic ACTH syndrome were extracted by the method of Island and coworkers (7). Homogenization time was limited to about 5 min, and heating was not employed. "MSH" was extracted from plasma (10–15 ml) with 4 volumes of acetone–12 N HCl (40 : 1 v/v) on ice for 15 min (14). The resultant supernatant was immediately washed twice with ethyl ether for 3 min according to the method of Yalow and coworkers (15). The volume of ethyl ether was 6 and 4 times that of original plasma, respectively. The aqueous phase was further purified in Amberlite CG-50 by the method of Island and coworkers (7).

Biologic assays of melanocyte-stimulating activity were performed by the in vitro frog skin-darkening method of Shizume and coworkers (16).

Results

Sensitivity of β-MSH radioimmunoassay. One antiserum was selected on the basis of its sensitivity for β-MSH. This particular antiserum was

[3] The normal subjects were pretreated overnight with 4 mg of dexamethasone, and only plasma that afforded excellent separation of the bound β_h-MSH-^{131}I from the free β_h-MSH-^{131}I was used as a carrier.

obtained from an animal that had received a total of 15 injections of commercial ACTH, 40 U each week, without Freund's adjuvant. A standard curve with this antiserum at a dilution of 1 : 10,000 is depicted in Fig. 2. In this system it was consistently possible to detect as little as 0.001 ng of β_h-MSH by means of statistically significant displacement of labeled β_h-MSH from the antibody. To construct displacement curves of sufficient extent to permit an evaluation of the parallelism of slopes of "unknown" extract and standard β_h-MSH, at least 0.02 ng of β-MSH was required.

Specificity of β-MSH radioimmunoassay (Fig. 3). Since the β-MSH used in immunization was "contaminated" with ACTH and perhaps other proteins, it seemed probable that the antiserum would contain a variety of antibodies. It was necessary, therefore, to use pure β_h-MSH both as labeled and unlabeled standard antigen to assure specificity of the radioimmunoassay.

Several synthetic analogues of MSH and ACTH were tested for their capacity to cross-react with the β-MSH antiserum and displace β_h-MSH–^{131}I from the bound to the free portion of the chromatoelectrophoretogram. α-MSH, α^{1-10}-ACTH, α^{1-24}-ACTH, $\alpha^{7-13(NH_2)}$-ACTH, α^{11-24}-ACTH, *N*-acetyl-α^{1-24}-ACTH, and $\alpha_p{}^{25-39}$-ACTH all failed to displace β_h-MSH–^{131}I, even when quantities as large as 500 ng were added to the incubation mixture.

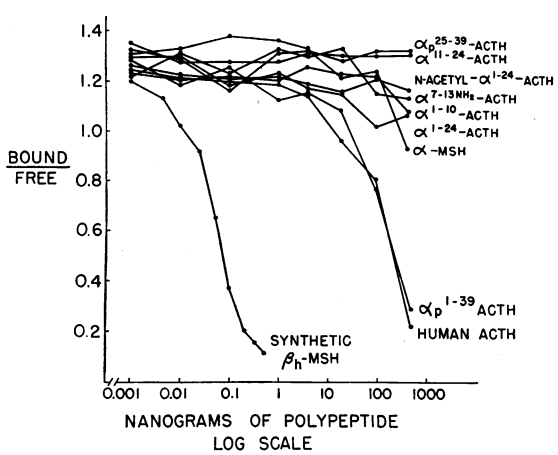

FIG. 3. TESTS FOR CROSS-REACTIVITY OF MSH AND ACTH ANALOGUES WITH THE β-MSH ANTISERUM. The quantity of each polypeptide is noted on the abscissa. The nomenclature of the analogues follows the convention suggested by C. H. Li (17).

Highly purified human ACTH and synthetic porcine ACTH did displace labeled β_h-MSH from the antibodies, but these polypeptides were only one-three thousandth as effective as β_h-MSH itself. The plasma and tissue extracts used in the present study were bioassayed for ACTH activity to exclude the possibility that ACTH could be present in quantities great enough to introduce an error in the β-MSH radioimmunoassay.

Highly purified porcine β-MSH was as active as synthetic β_h-MSH in displacing labeled β_h-MSH from the antibodies, but there is no evidence that porcine β-MSH occurs in man. Therefore, since the starting material in all of our studies was human tissue or plasma and since significant interference could not be ascribed to α-MSH, ACTH, or any of their available analogues, it seemed reasonable to assume that the material detected by the radioimmunoassay was either human β-MSH or a very closely related analogue. Extracts of tissues other than pituitary glands or tumors of patients with the ectopic ACTH syndrome did not contain detectable quantities of β-MSH by radioimmunoassay.

Recovery of β-MSH. Plasma from two normal subjects was divided into several 10-ml portions. Three portions of each were extracted and subjected to radioimmunoassay for β-MSH. The assay results from the first subject were 0.042, 0.042, and 0.055 ng/ml (mean 0.046), and those for the second were 0.078, 0.084, and 0.090 ng/ml (mean 0.084). To other portions of the same plasma, known quantities of β_h-MSH were added before extraction and assay. The results of these assays are shown in Table I. In nine assays the recovery of β-MSH ranged from 59 to 81% of the amount present in plasma before extraction.

Assays of human pituitary extracts. Two individual human pituitary glands were extracted and subjected to bioassay and radioimmunoassay. Radioimmunoassay results are plotted in Fig. 4. The parallelism between the displacement curves of the pituitary extracts and that of standard β_h-MSH indicates that the extracts contained a substance that was immunologically indistinguishable from β-MSH. The concentrations of β-MSH in these two human pituitaries were 336 and 357 μg/g of wet pituitary tissue, respectively. Bioas-

TABLE I

Recovery of β-MSH from plasma

Sample	Assay No.	β_h-MSH Expected	β_h-MSH Found	Recovery
		ng/ml		%
Plasma of subject 1 plus β_h-MSH, 0.05 ng/ml	1	0.096	0.066	69
	2		0.072	75
	3		0.078	81
Plasma of subject 1 plus β_h-MSH, 0.5 ng/ml	4	0.546	0.32	59
	5		0.34	62
	6		0.38	70
Plasma of subject 2 plus β_h-MSH, 5 ng/ml	7	5.084	3.2	63
	8		3.2	63
	9		3.4	67
Average				68

says of the same extracts gave estimates of 348 and 372 μg/g of wet pituitary tissue respectively, with synthetic β_h-MSH as a bioassay reference standard.[4] It appears, therefore, that most of the biologic melanocyte-stimulating activity of human pituitary extracts can be accounted for by their content of β-MSH.

Assays of human tumor extracts (Fig. 4, Table II). Tumor tissues of 11 patients with the ectopic ACTH syndrome were extracted and subjected to bioassay and radioimmunoassay. The parallelism between the displacement curves representing tumor extracts and that of standard β_h-MSH indicated that each tumor contained a substance that was immunologically indistinguishable from β-MSH. In the radioimmunoassay the apparent concentrations of β-MSH in various tumors ranged from 3 to 1600 ng/g of wet tumor tissue, accounting for 25–140% of the biological MSH activity.

Assays of plasma from hyperpigmented patients with Addison's disease, Cushing's disease (post-adrenalectomy), and the ectopic ACTH syndrome (Fig. 4, Table III). Specimens of plasma of three patients with untreated Addison's disease, seven patients who had undergone adrenalectomy as treatment for Cushing's disease, and two patients with the ectopic ACTH syndrome were extracted and subjected to bioassay and radioimmunoassay. Each plasma extract contained a substance that was immunologically indistinguishable from

[4] Synthetic β_h-MSH has a biologic potency of approximately 3.3×10^9 U/g, expressed in the units employed by Shizume, Lerner, and Fitzpatrick (16).

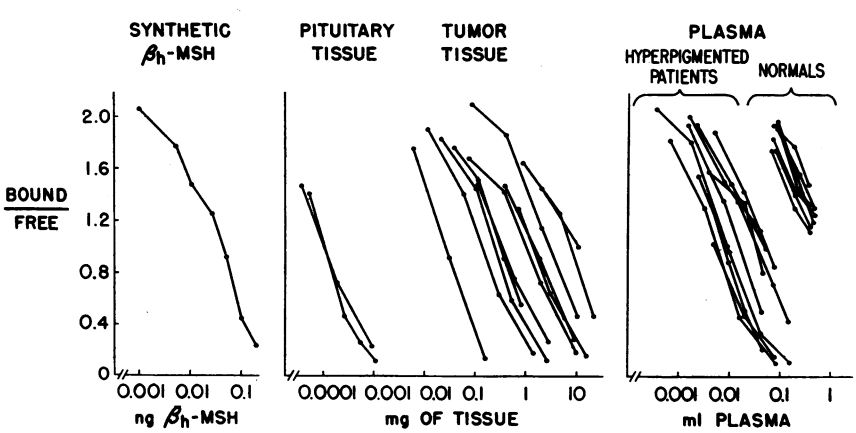

FIG. 4. RADIOIMMUNOASSAY OF β-MSH IN HUMAN PITUITARY GLANDS, IN TUMORS FROM PATIENTS WITH THE ECTOPIC ACTH SYNDROME, IN PLASMA FROM HYPERPIGMENTED PATIENTS, AND IN PLASMA OF NORMAL SUBJECTS.

TABLE II

β-MSH concentrations of tumors of the patients with the ectopic ACTH syndrome

Patient	Immunologic β-MSH	Biologic ACTH activity*
	ng/g	*ng/g*
C.O.	25	34
I.P.	30	34
D.H.	258	210
J.N.	126	90
J.S.	14	18
L.G.	6	18
V.B.	102	402
D.S.	1600	1860
C.W.	25	96
J.G.	108	216
F.T.	3	3

* Biologic MSH activity expressed as nanograms of synthetic β_h-MSH.

β-MSH. Again, most of the biologic MSH activity of the plasma extracts could be accounted for by β-MSH. The apparent concentration of β-MSH in various specimens ranged from 0.5 to 6 ng/ml of plasma. By way of contrast, in seven patients with well-controlled Addison's disease, plasma β-MSH values were only 0.03–0.11 ng/ml.

Assays of β-MSH in plasma of normal subjects (Fig. 4). Extracts of plasma from seven normal subjects also displaced labeled β_h-MSH from antibodies with displacement curves parallel to those obtained with standard unlabeled synthetic β-MSH, but the extent of the radioimmunoassay was limited to three points for each extract, since the incubation system could accommodate only 0.02 ml of extract representing 0.6 ml of plasma. Use of larger quantities of plasma extract resulted in poor resolution of bound and free peaks. Starting with large quantities of normal plasma, we assayed two extracts both in the isolated frog skin system and by radioimmunoassay; the results of bioassays agreed closely with those obtained by radioimmunoassay. The apparent concentrations of β-MSH in plasma of nomal human subjects were less than 0.09 ng/ml.

Correlation between degree of hyperpigmentation and concentration of β-MSH in plasma (Fig. 5). An attempt was made to evaluate the clinical significance of plasma β-MSH in patients with "addisonian" hyperpigmentataion. A clinician without knowledge of the assay results assigned semiquantitative estimates (0 to 4 +) of the degree of hyperpigmentataion, based on each patient's history and physical examination. These estimates were depicted on the ordinate of a graph, the abscissa of which represented the concentration of β-MSH in the plasma as determined by radioimmunoassay. The results revealed a distinct positive correlation between the two measurements.

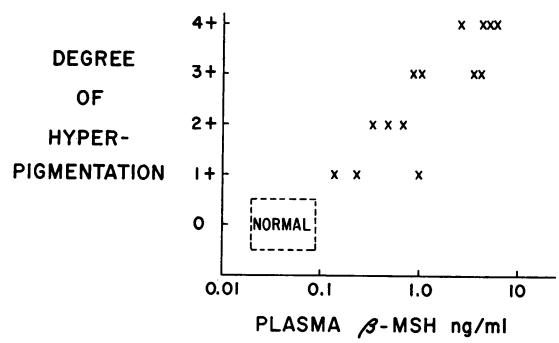

FIG. 5. CORRELATION OF CLINICAL HYPERPIGMENTATION WITH THE CONCENTRATION OF β-MSH IN THE PLASMA.

TABLE III

β-MSH concentration in the plasma of three patients with Addison's disease, seven patients previously adrenalectomized for Cushing's disease, and two patients with the ectopic ACTH syndrome

Disease	Patient	Immunologic β-MSH	Biologic MSH activity*
		ng/ml	*ng/ml*
Addison's disease	J.F.	0.5	0.5
	G.S.	0.8	0.9
	J.R.	1.1	0.6
Cushing's disease	A.W.	0.7	0.6
	E.K.	3.7	1.7
	H.R.	3.9	4.3
	E.B.	2.7	5.0
	M.J.	5.2	3.8
	G.K.	6.0	2.0
	B.H.	1.1	0.7
Ectopic ACTH syndrome	M.J.	5.1	3.4
	J.L.	0.9	0.3

* Biologic MSH activity expressed as nanograms of synthetic β_h-MSH.

Discussion

In 1959, Harris elucidated the amino acid sequence of β-MSH from human pituitary glands (10). Porcine β-MSH is similar to human β-MSH except that it has 18 amino acids instead of 22 and contains lysine instead of arginine at position 6. This similarity of amino acid sequence suggested that antibodies to porcine β-MSH might

be useful in measuring human B-MSH by radioimmunoassay. Since the β-MSH preparation used in the production of antisera also contained other pituitary hormones, it was important to have pure β-MSH as a test antigen. Fortunately, the antiserum clearly distinguished β-MSH from α-MSH and ACTH, and this was important because these two polypeptides do occur in man and possess biologic MSH activity (9).

Sensitive and specific radioimmunoassays have been needed to complement information obtained with the relatively nonspecific bioassay method. As in any immunologic assay, the specificity of the present method depends upon the validity of the assumption that "specific" β-MSH antibodies do not cross-react to a significant degree with other compounds that might occur in the mixture to be assayed. For practical purposes, this appears to be true. Polypeptide-rich mixtures that are known to be devoid of MSH activity have failed to cause any displacement of labeled β-MSH from the antibodies used in the present radioimmunoassay system. Furthermore, the known structural analogues of β-MSH that would seem most likely to occur in human tissues (α-MSH and ACTH fragments) also failed to cross-react with β-MSH antibodies to such an extent that they would be mistaken for β-MSH. A compound that behaves like β-MSH in this system is in all probability either β-MSH or a very closely related analogue.

The MSH activity of plasma has previously been shown to be abnormally high in patients with Addison's disease (7, 8, 16), in patients who have developed hyperpigmentation after bilateral adrenalectomy as treatment for Cushing's disease (7–9), and in patients with hyperpigmentataion associated with the ectopic ACTH syndrome (7–9). In each of these conditions, plasma ACTH is characteristically elevated. The quantity of ACTH, however, is far too small to account for the amount of melanocyte-stimulating activity found by bioassay (7–9). Furthermore, the major melanotropin of the plasma of such patients has been shown to be physically separable from the major plasma adrenocorticotropin (7, 8). Therefore, although ACTH does have intrinsic melanocyte-stimulating activity, it does not appear to be the major pigmentary hormone of human plasma.

A similar situation has been described with regard to α-MSH (9). This hormone was identified and quantified by radioimmunoassay in human pituitary tissue and in tumors of patients with the ectopic ACTH syndrome, but the quantities of α-MSH were too small to account for more than a minor fraction of the total biologic MSH activity in these tissues. α-MSH has yet to be identified in the plasma of either normal or hyperpigmented subjects.

The present study has shown that a third melanotropin, β-MSH, is demonstrable in the plasma of each of the three disorders characterized by addisonian hyperpigmentation. In most cases, the quantities of total MSH as estimated by radioimmunoassay were in fairly close agreement with the quantities of total MSH as estimated by biologic assay. It seems probable, therefore, that the major pigmentary hormone in the plasma of patients with addisonian hyperpigmentataion is β-MSH.

Some of the tumor and plasma extracts did not show close agreement between the results of the bioassay and the results of the radioimmunoassays for β-MSH. A number of explanations are possible. First, neither assay method is perfectly precise. The coefficient of variation for the bioassay has been found to be 23%, and that for the radioimmunoassay has been found to be 13% in our laboratory. Some of the discrepancies, then, could be due merely to the variances of the methods. Second, some of the specimens (especially the tumors) might have undergone degenerative changes that could have affected either their biologic potency or their immunologic reactivity to some degree. Or, third, some of the specimens might originally have contained either a significant quantity of a melanocyte-stimulating factor other than β-MSH or a significant quantity of a biologically inactive cross-reactant with β-MSH antibodies. Nevertheless, in most extracts most of the biologic melanocyte-stimulating activity could be accounted for by the quantity of β-MSH found by radioimmunoassay.

The close biologic relationships among ACTH, α-MSH, and β-MSH can be appreciated from consideration of the fact that all of these hormones have been found to accompany each other not only in pituitary tissue but also in tumors associated with the ectopic ACTH syndrome, even

though the tumors themselves arose from a variety of tissues of origin. One might speculate that the synthesis and secretion of these three polypeptides might be controlled by a common mechanism.

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