ISOLATION OF MELANOCYTE-STIMULATING HORMONE
FROM HOG PITUITARY GLAND*

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The present communication is concerned with two substances possessing melanocyte-stimulating hormone (MSH) activity, α-MSH and β-MSH, found in hog pituitary gland. The main component, α-MSH, was isolated by a four-step process consisting of solvent extraction and fractional precipitation, oxycellulose adsorption, counter-current distribution, and paper electrophoresis. α-MSH has been found to be homogeneous with respect to counter-current distribution and to paper electrophoresis at different pH values. A preliminary report on the isolation of α-MSH appeared elsewhere (1). These hormones, which are produced in the intermediate lobe of the pituitary gland (2), previously were called the melanophore hormone, melanophore-expanding hormone, or intermedin. It has been known for several years that the skin of aquatic animals could be darkened by injections of these hormones (3). Recently, a similar effect was observed in human beings (4).

EXPERIMENTAL

Method of Assay—MSH was determined by an assay in vitro by using isolated skin from Rana pipiens frogs (5). The changes in skin color before and after addition of MSH were measured with a photoelectric reflection meter. Within limits the logarithm of MSH concentration was proportional to the ratio of the changes in reflectance obtained with an unknown and standard quantity of hormone. A unit of MSH is defined as the degree of darkening produced by 0.04 γ of a lyophilized water extract of beef posterior pituitary powder on isolated frog skin (5).

Starting Material—Early in the investigation it was recognized that acetone-dried posterior pituitary powder from hogs had approximately twice the MSH activity as that from cows. Although it was not known

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whether this was due to a different concentration of MSH or to a variation in chemical structure of the hormones from the two sources, it was decided to use the more potent material from hogs as the starting substance. Posterior pituitary powder had about 10 times the MSH activity as that from the anterior portion of the gland.

Preliminary studies of crude MSH preparations showed rapid dialysis of the active material through cellophane and collodion membranes. Hormonal activity was lost when MSH was mixed with undistilled solvents such as diethyl ether or butyl alcohol. The ninhydrin reaction resulted in complete loss of activity. Treatment of MSH with 0.1 N sodium hydroxide in a boiling water bath for 5 minutes modified its darkening properties. The alkali-treated material produced more prolonged darkening than the untreated preparation.

Isolation of MSH—The methods described below were used in the isolation of α-MSH.

The first step was a modification of the procedure devised by Payne et al. (6) for the extraction of corticotropin (ACTH). 100 gm. of hog posterior pituitary powder containing $3 \times 10^7$ MSH units per gm. were mixed with 20 ml. of acetone to give a smooth paste. 1 liter of glacial acetic acid was added and the mixture was heated on a hot-plate at 50° for 10 minutes with constant stirring. The supernatant fluid was removed after centrifugation, and the residue was extracted again with 1 liter of glacial acetic acid in the same manner except that the heating at 50° was omitted. The combined supernatant fluids (1900 ml.) were mixed with 0.5 volume of acetone (950 ml.) and then with 10 ml. of saturated sodium chloride solution. After remaining at room temperature for 15 to 20 minutes the mixture was centrifuged. The supernatant fluid (2700 ml.) was mixed with 2 volumes (5400 ml.) of cold petroleum ether and allowed to stand overnight at $-10^\circ$. Two layers formed: a heavy precipitate settled as a hard cake at the bottom of the flask, and small amounts of precipitate collected at the interface. The fluid was decanted, and the precipitate adhering to the bottom of the flask was covered immediately with acetone. If addition of acetone were delayed, the precipitate formed a gummy mass which was difficult to manipulate. After several washings with acetone, a fine yellow powder resulted which could be dried readily in vacuo. Usually 16 to 18 gm. of product containing 1 to $2 \times 10^8$ MSH units per gm. were obtained. The yield with respect to MSH activity was approximately 85 per cent. This petroleum ether-precipitated fraction will be referred to as PEF.

8 gm. of PEF were dissolved in 160 ml. of 0.1 N acetic acid by gentle warming in a boiling water bath. An insoluble residue was removed by centrifugation at 18,000 $\times g$. 16 gm. of washed oxycellulose (7) were
added to the supernatant fluid, and the mixture was shaken gently for 75 minutes and then centrifuged. The supernatant fluid was discarded. The oxycellulose was washed two or three times with 0.1 N acetic acid and then the active material was eluted by shaking the oxycellulose with 100 ml. of 80 per cent acetic acid for 60 minutes. After centrifugation the supernatant fluid was removed and centrifuged again at 18,000 × g to remove the last traces of oxycellulose. The separated portions of oxycellulose were saved for re-elution in large batches to recover additional active material. The supernatant fluid was diluted with an equal volume of distilled water and lyophilized. The dilution step was necessary because solutions containing more than 40 but less than 100 per cent acetic acid could not be lyophilized with our equipment. Approximately 0.5 gm. of a pale yellow solid containing 1 to 1.5 × 10⁹ MSH units per gm. was obtained. The MSH yield was approximately 50 per cent. This oxycellulose fraction was designated OXF.

In preparation for counter-current distribution, 2 gm. of OXF were dissolved in a mixture of 75 ml. of each phase of the solvent system, sec-butyl alcohol-0.5 per cent aqueous trichloroacetic acid, with the aid of a micro-Waring blender at about -10°. The solvents were prepared by equilibrating equal volumes of 0.5 per cent aqueous trichloroacetic acid and redistilled sec-butyl alcohol at 5° for 24 hours. The resultant solution gradually separated into two layers and was used as the initial tube of a twelve tube distribution unit at 5°, with 75 ml. of each solvent phase being used per tube. The distribution proceeded with the lower phase advancing. MSH analysis of various fractions indicated that tubes 3 to 5 contained the major portion of MSH activity (Fig. 1). After completion of the run, contents of tubes 3 to 5 were combined and lyophilized. Traces of trichloroacetic acid left with the solid after lyophilization were removed immediately by repeated extraction with freshly distilled diethyl ether. At no time during this extraction was a given amount of ether allowed to remain in contact with the solid for more than a minute. The resultant light brown solid weighed 0.5 to 0.6 gm. and had an activity of 3 to 4 × 10⁹ MSH units per gm. The MSH yield was approximately 75 per cent. This counter-current distribution fraction was designated CDF.

An electrophoretic apparatus with a 50 cm. horizontal migration path of the type described by Kunkel and Tiselius (8) was used to purify Fraction CDF further. The power supply had a capacity of 0 to 1000 volts and 0 to 50 ma. Whatman No. 3 MM filter paper strips, 11 × 58 cm., were used as the supporting medium. Fraction CDF was subjected to paper electrophoresis as follows: Pyridinium acetate buffer, pH 4.55, was prepared by mixing 2 volumes of 0.3 N acetic acid and 1 volume of 0.3 M aqueous pyridine solution. 40 mg. of CDF were dissolved in 0.6 ml. of
0.3 N acetic acid by warming slightly in a water bath. To the mixture was added 0.3 ml. of 0.3 M pyridine solution. The small amount of residual solid was removed by centrifugation of the solution at 18,000 X g. The supernatant fluid was applied dropwise to a filter paper strip. Great care was taken to confine the applied solution to a band no more than 1 cm.

**Fig. 1.** Twelve tube counter-current distribution of Fraction OXF in sec-butanol-0.5 per cent aqueous trichloroacetic acid system at 5°. Curve A represents weight of solid per tube. Curve B represents MSH activity of solid per tube. This preparation was obtained after solvent extraction and fractional precipitation of pituitary powder followed by oxycellulose adsorption and elution.

**Fig. 2.** Paper electrophoresis of Fraction CDF in pyridinium acetate buffer at pH 4.55, 18 volts per cm. of migration path, for 11 hours at 5°. The arrow indicates the site of application of the hormone. The crosses on the upper portion of the diagram indicate the location of prominent spots revealed by bromophenol blue staining. Fraction CDF was prepared by solvent extraction and fractional precipitation of pituitary powder, oxycellulose adsorption, and counter-current distribution in a twelve tube counter-current unit at 5°.

The electrophoretic run usually lasted 10 to 12 hours at a potential of 18 to 20 volts per cm. of migration path and a current of 8 to 10 ma.

After completion of electrophoresis, the paper was frozen immediately to facilitate handling. A strip 1 cm. in width was cut along the whole length of the filter paper and dried for 5 to 10 minutes in an oven at 90°. After thorough drying the paper strip was made strongly alkaline by passage back and forth over a flask containing concentrated ammonium hydroxide. It was then stained for 30 seconds with 1 per cent bromophenol blue in 95 per cent alcohol saturated with mercuric chloride. The strip
was washed with a fine stream of 0.5 per cent acetic acid until all excess dye was removed. An electrophoretic pattern appeared as gray-blue bands which were converted to an intense blue color in the presence of ammonia vapor. The main MSH activity was associated with the second band located approximately 20 cm. from the origin in the direction of the cathode (Fig. 2).

The stained strip was used as a guide so that the active fraction could be cut from the remainder of the filter paper. MSH was eluted by shaking with 50 ml. of 3 N acetic acid for 60 minutes at 5°. The material was filtered and lyophilized immediately. Approximately 4 mg. of a spongy white solid having an activity of 1 to 2 \( \times 10^{10} \) MSH units per gm. were obtained. The MSH yield was about 40 per cent. This highly potent fraction was referred to as HPF-1.

An alternative procedure to the last step was tried in which Fraction CDF was submitted to two consecutive electrophoretic runs with an alkaline buffer in the first and an acidic buffer in the second. The electrophoretic runs were carried out as follows: 40 mg. of fraction CDF were dissolved in 1 ml. of sodium barbital buffer and submitted to electrophoresis in the same manner as previously described. Sodium barbital buffers prepared according to Michaelis (9) with pH values ranging from 8 to 9 were found effective. The active material was associated with the second band located approximately 12 cm. from the origin in the direction of the cathode.
cathode (Fig. 3) and was isolated as described above. After lyophilization
the entire fraction was dissolved in 1 ml. of 0.2 N acetic acid for electrophoresis in the pyridinium acetate buffer at pH 4.55. Only a single band could be visualized in the stained guide strip. MSH analysis of different segments of the filter paper and ninhydrin tests on similar samples after acid hydrolysis showed single peaks which overlapped completely (Fig. 4). Extraction of the active region yielded about 1 mg. of a white spongy solid with an activity of 1 to 2 \times 10^{10} MSH units per gm. The over-all yield

![Fig. 4. MSH activity and ninhydrin color reaction of Fraction CDF after two paper electrophoretic runs. The active fraction was removed from the run at pH 8.0, 18 volts per cm. of migration path, for 8 hours and then tested at pH 4.55, 18 volts per cm., for 12 hours. Both runs were done at 5°. Curve A represents MSH activity. Curve B represents ninhydrin color reaction. The arrow indicates the site of application of the hormone.](http://www.jbc.org/)

of MSH in these two runs was approximately 10 per cent. This highly potent fraction was referred to as HPF-2.

*Homogeneity of Purified MSH*—The MSH preparations, HPF-1 and HPF-2, obtained after one and two electrophoretic runs, respectively, were tested for homogeneity by electrophoretic analysis with buffers ranging in pH from 1.40 to 12.2. The latter included glycine-sodium chloride-hydrochloric acid, pH 1.40, \( \mu = 0.1 \); pyridinium acetate-acetic acid, pH 4.55, \( \mu = 0.1 \); sodium barbital-sodium acetate-hydrochloric acid, pH 8.9, \( \mu = 0.05 \); and glycine-sodium chloride-sodium hydroxide, pH 11.3, \( \mu = 0.04 \); pH 11.9, \( \mu = 0.1 \); and pH 12.2, \( \mu = 0.1 \). The techniques used for electrophoresis were outlined previously. At all levels of pH tested, the hormone behaved as if it were a single component. In the electrophoretic run in which glycine buffer at pH 11.3 was used, MSH migrated only a
very short distance toward the cathode, being similar in this respect to dextran spots applied on both sides of the test material. Therefore, the isoelectric point of MSH was estimated to be within the range pH 10.5 to 11.0.

Homogeneity tests were carried out with a 100 tube, all glass counter-current distribution apparatus at room temperature (24 to 26°) with the upper phase advancing. The solvent system used was sec-butyl alcohol and 0.5 per cent aqueous trichloroacetic acid equilibrated at room temperature. At the end of a run the upper and lower phases were removed separately from each cell. The optical density at a wave-length of 2750 Å

![Fig. 5. Counter-current distribution of 20 mg. of Fraction HPF-1 in sec-butanol-0.5 per cent aqueous trichloroacetic acid system at room temperature; X, experimental points based on weight of solid per 10 ml. of upper phase; O, theoretical points; Δ, experimental points based on MSH activity per ml. of upper phase. This preparation was obtained from paper electrophoresis of Fraction CDF.](http://www.jbc.org/)

and the MSH activity were measured for each tube. The amount of solid in mg. per 10 ml. of upper phase was calculated from the optical density.

20 mg. of HPF-1 were distributed through 97 transfers during a 6 hour period. Comparison of the experimental distribution curve based on weight of solid per 10 ml. of upper phase with the theoretical curve calculated according to the usual procedure (10) revealed that approximately 95 per cent of the MSH was a single component (Fig. 5). The MSH activity curve was symmetrical and gave the same peak as the curve based on mg. of solid per 10 ml. of upper phase. From the position of the peak the distribution coefficient for MSH in the two solvents was calculated to be 2.1. Only about 50 per cent of the MSH used at the beginning of the experiment could be accounted for by the end of the run on the basis of MSH activity. When MSH was allowed to stand for 6 hours at room tempera-
ture in a test-tube containing the solvents used in the counter-current run, marked loss in activity occurred. Unfortunately, facilities were not available for carrying out the distribution at 5°.

The MSH activity curve was narrower than the theoretical curve or the curve based on weight of solid per 10 ml. of upper phase. This finding may have resulted from a greater loss of MSH activity on a percentage basis in the dilute tubes than in the concentrated tubes during the counter-current run. On the other hand, the rate of decrease in ultraviolet light absorption with time was the same in all tubes.

Fig. 6. Counter-current distribution of 100 mg. of Fraction CDF in sec-butanol-0.5 per cent aqueous trichloroacetic acid system at room temperature. Curve A represents optical density of upper phase at λ = 2750 A. Curve B represents MSH activity of upper phase. This preparation was obtained after counter-current distribution of Fraction OXF in a twelve tube counter-current unit at 5°.

Since the distribution curve was determined from the optical density and not on the basis of dry weight of solid per cell, it was possible that the preparation contained impurities which did not absorb light at the wavelength used. To clarify this point, samples from every fifth tube were subjected to acid hydrolysis for 10 to 12 hours and then allowed to react with ninhydrin. Only one ninhydrin-positive peak corresponding to the ultraviolet absorption and MSH activity peaks was found.

5 mg. of HPF-2 were examined by counter-current distribution in the same way through forty-nine transfers. Similar results were obtained. The calculated distribution coefficient of MSH in the two solvents was in exact agreement with the previous results.

Counter-Current Distribution and Moving Boundary Electrophoresis of Fraction CDF—When Fraction CDF was distributed through 97 transfers
in the large counter-current apparatus at room temperature, at least three peaks were found by ultraviolet absorption (Fig. 6). However, only one corresponded to a peak of great MSH activity. This occurred at the same position as the MSH peak of the purified hormone, Fractions HPF-1 and HPF-2 (Fig. 5), as well as that obtained with crude posterior pituitary powder (Fig. 8). A second relatively small peak also was obtained from Fraction CDF.

![Fig. 7. Moving boundary electrophoresis of Fraction CDF in pyridinium acetate buffer, pH 4.55, 225 volts, 20 ma., for 173 minutes and 8 seconds at 2°. The arrows indicate the direction of migration.](image)

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Part of electrophoretic cell</th>
<th>Component (see Fig. 7)</th>
<th>Potency of solution</th>
<th>Potency of solid</th>
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</thead>
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<tr>
<td>a1</td>
<td>Ascending limb</td>
<td>1</td>
<td>$0.15 \times 10^6$</td>
<td>0.2</td>
</tr>
<tr>
<td>a2</td>
<td>&quot;</td>
<td>$1 + 2$</td>
<td>$5.4 \times 10^6$</td>
<td>5.0</td>
</tr>
<tr>
<td>a3</td>
<td>&quot;</td>
<td>$1 + 2 + 3$</td>
<td>$7.2 \times 10^6$</td>
<td>3.0</td>
</tr>
<tr>
<td>a4</td>
<td>&quot;</td>
<td>$1 + 2 + 3 + 4$</td>
<td>$8.7 \times 10^6$</td>
<td>1.6</td>
</tr>
<tr>
<td>d3 + 4 + 5</td>
<td>Descending &quot;</td>
<td>$3 + 4 + 5$</td>
<td>$6.7 \times 10^6$</td>
<td>0.3</td>
</tr>
<tr>
<td>Bottom</td>
<td>Bottom</td>
<td>&quot;</td>
<td>$8.5 \times 10^6$</td>
<td>Not determined</td>
</tr>
<tr>
<td>Original</td>
<td>&quot;</td>
<td>&quot;</td>
<td>$2.3 \times 10^7$</td>
<td>4.0</td>
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</table>

In addition 100 mg. of Fraction CDF were studied by moving boundary electrophoresis in a standard electrophoretic cell at 2° with a pyridinium acetate buffer at pH 4.55, $\mu = 0.1$. Whereas only three peaks were found after counter-current distribution, electrophoresis showed five components with different mobilities moving toward the cathode (Fig. 7). A sampling device designed by Rigas et al. (11) was used to remove the solutions in the electrophoretic cell. Six separate fractions containing mixtures of the various components were obtained. These solutions were lyophilized and both the liquid and lyophilized powder were assayed for MSH activity. Results given in Table I show that the second peak possessed the main MSH activity and that this fraction corresponded to the active MSH obtained by paper electrophoresis.
Stability of MSH—It had been assumed that MSH was a relatively stable molecule because posterior pituitary powder could be kept at room temperature for an indefinite period without loss of activity. Furthermore, when mixtures of MSH were heated with dilute alkali, MSH activity was retained, whereas that of the other polypeptide hormones present was destroyed. Hence, we were surprised to find that highly purified MSH was very unstable. In order to determine the best conditions for storage, dry samples of the active hormone, HPF-1, were kept in sealed vials under a variety of conditions. As shown in Table II, it was found that MSH could be kept at \(-17^\circ\) under nitrogen, oxygen, or \(\textit{in vacuo}\) for 19 days with some, but not marked, loss of activity. However, under \(\text{H}_2\text{S}\), the loss in potency during a similar period was appreciable. Storage for 50 days under air and

<table>
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<tr>
<th>Sample No.</th>
<th>Atmosphere</th>
<th>Storage temperature</th>
<th>Time of storage</th>
<th>Potency after storage</th>
<th>Per cent of original potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vacuum</td>
<td>-17</td>
<td>19</td>
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</tr>
<tr>
<td>2</td>
<td>Nitrogen</td>
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<tr>
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<tr>
<td>4</td>
<td>(\text{H}_2\text{S})</td>
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<td>19</td>
<td>2.0</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
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<td>19</td>
<td>1.4</td>
<td>11</td>
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<tr>
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<tr>
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<td>50</td>
<td>3.5</td>
<td>22</td>
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</table>

\(\textit{in vacuo}\) also caused a decrease in potency. The hormone was relatively unstable under oxygen at room temperature. Samples 6 and 7 were prepared in detergent-free vessels at the stage of electrophoresis, but glassware used at other times had been washed with detergents and rinsed well. No significant difference was observed.

Main MSH Component of Pituitary Gland—The following yields of MSH activity resulted from the four-step isolation process: solvent extraction and fractional precipitation, 85 per cent; oxycellulose adsorption, 50 per cent; counter-current distribution, 75 per cent; and paper electrophoresis, 10 to 40 per cent. Although the yield of MSH in each step was relatively high, the over-all yield was between 5 and 15 per cent. Thus, the question arose as to whether or not the hormone finally isolated represented the main MSH component of hog posterior pituitary gland. To help decide this point, posterior pituitary powder from hogs was distributed through 97 transfers in the counter-current distribution apparatus by using the same solvent system described previously. 1 gm. of hog pituitary powder was
shaken with a mixture of 20 ml. of each solvent phase at room temperature for 30 minutes. The insoluble residue was removed by centrifugation. 10 ml. of each phase of the extract were mixed and charged to the first tube of the counter-current distribution apparatus. The material was distributed with 10 ml. of each solvent phase through 97 transfers. The distribution curve based on MSH activity per tube as given in Fig. 8, Curve A, indicated the presence of two active components, which for simplicity were designated α-MSH and β-MSH. α-MSH (K = 2.1) and β-MSH (K = 0.6) represented about three-fourths and one-fourth, respectively, of the total MSH activity of the contents of all tubes. More than 70 per cent of the total activity present in the pituitary powder (0.5 gm.) was accounted for. These same components were observed in the distribution curves of the partially purified preparations, CDF and OXF, as indicated in Figs. 6 and 9, Curve B. Since the purified hormone preparation, HPF-1, and a partially purified fraction prepared according to the procedure of Landgrebe and Mitchell (12) each contained only one active component, α-MSH and β-MSH, respectively (Figs. 5 and 9, Curve A), it is unlikely that one of the active components was an artifact produced by the solvent system. Therefore, it can be assumed that two distinct melanocyte-stimulating
hormones are present in hog pituitary gland. When anterior pituitary powder from hogs was distributed in the same manner, α-MSH and β-MSH were observed in the same ratio and were characterized by the same distribution coefficient (Fig. 8, Curve B), as was the case with posterior pituitary powder, although there was a 10-fold difference in the total MSH activity. The striking resemblance between the two distribution curves suggests that these two melanocyte-stimulating hormones probably share a common site of synthesis in the pituitary gland, presumably the intermediate lobe.

Fig. 9. Counter-current distribution runs of 16 mg. of partially purified MSH (Curve A) prepared according to Landgrebe and Mitchell (12) and 30 mg. of Fraction OXF (Curve B) in sec-butanol-0.5 per cent trichloroacetic acid at room temperature.

α-MSH has a specific potency of 1 to 2 × 10¹⁰ MSH units per gm. when purified by the procedure described previously. We have not determined the specific potency of β-MSH. It might be difficult to determine the relative potency of these two hormones because both are unstable and the measured potencies may not represent true values. The electrophoretic properties of inactivated MSH are identical with those of the active hormone. Thus any inactivated MSH present in preparations used as starting material for the final paper electrophoresis would appear in the end-product and cause undue lowering of specific potency. Hence, these two hormones must be prepared under identical conditions in order to compare their activities. Regardless of the specific activity of α-MSH, it is the main MSH component of hog pituitary gland, because the total α-MSH activity is greater than that of β-MSH.
In the past several reports have been made on purification of MSH from pituitary glands (12–16). In most cases the purified substance showed a moderate, but not marked, increase in potency over the starting material. However, recently Porath et al. (17) isolated a peptide which was homogeneous by ultracentrifugation and electrophoresis. It had about 1000 times the MSH activity of hog posterior pituitary powder, an isoelectric point at pH 5.2, and an approximate molecular weight of 3000. Geschwind and Li² reported isolation of a similar homogeneous preparation possessing a specific potency of 1 to 5 × 10⁹ MSH units per gm., an isoelectric point at pH 5.5, and a distribution coefficient of 0.6 in the system sec-butanol-0.5 per cent trichloroacetic acid at 20°. Benfey and Purvis (18) also reported isolation of a highly purified fraction possessing 400 to 500 times the potency of the starting hog posterior pituitary powder. Their hormone preparation was similar in many respects to those prepared by Porath et al. and Geschwind and Li, except that it possessed a definitely basic isoelectric point.² These investigators apparently isolated β-MSH which accounts for less MSH activity in the pituitary gland than α-MSH.

In order to prove this point, a partially purified preparation, made from hog posterior pituitary powder according to the procedure described by Landgrebe and Mitchell (12),³ was subjected to counter-current distribution in the same solvent system that was used in our homogeneity studies. The distribution curve (Fig. 9, Curve A), based on MSH activity per tube, indicated a single peak located at tube No. 32 which corresponded to a distribution coefficient of about 0.5. This value was close enough to be considered in the same range as that of β-MSH because of the magnitude of error that may be involved in estimation of distribution coefficients from the distribution curve based on MSH activity. When the same material was subjected to paper electrophoresis in sodium barbital buffer at pH 8.9, we observed one active component migrating toward the anode with a mobility very similar to that of β-MSH (Fig. 3) and several inactive components migrating toward both electrodes. No active component with the characteristics of α-MSH was observed in either case, whereas at a similar stage of purification our preparation of OXF definitely contained two active components as shown in Fig. 9, Curve B. Since Landgrebe and Mitchell used acetone precipitation at pH 6.5 in their fractionation procedure, it was suspected that a substance with a highly basic isoelectric point like that of α-MSH would remain in solution. As a matter of fact, it was found that the lyophilized mother liquor contained far more total

¹ Unpublished data.
² J. L. Purvis, private communication.
³ This procedure was used by Porath et al. and also by Benfey and Purvis in their fractionation.
MSH activity than that of the active precipitate, although its specific potency was lower, owing to the dilution caused by the presence of large amounts of ammonium chloride. By means of paper electrophoresis, \( \alpha \)-MSH was found and identified as the only active component in the lyophilized filtrate. Evidently, \( \alpha \)-MSH was separated from \( \beta \)-MSH by acetone precipitation at pH 6.5 and was discarded in the filtrate during the fractionation. This was probably the reason that the other investigators isolated \( \beta \)-MSH as the main active component. Therefore, the original fractionation procedure of Landgrebe and Mitchell was modified as follows: The 0.1 N hydrochloric acid eluate from oxycellulose was lyophilized directly instead of being precipitated with acetone. The resultant solid was subjected to paper electrophoresis in the same buffer mentioned before. One basic and one acidic active component, as well as several inactive components, were observed. The mobilities of the basic and the acidic active

\[
\begin{array}{l|c|c}
\text{Amino acid} & \text{\( \alpha \)-MSH} & \text{\( \beta \)-MSH*} \\
\hline
\text{Aspartic acid} & 1.2 (1) \dagger & 4.0 (4) \dagger \\
\text{Glutamic acid} & 2.9 (3) & 3.8 (4) \\
\text{Serine} & 3.7 (4) & 2.0 (2) \\
\text{Glycine} & 3.1 (3) & 4.0 (4) \\
\text{Tyrosine} & 1.6 (2) & 0 \text{ (Tentative)} \\
\text{Lysine} & 3.2 (3) & 3.4 (3) \\
\text{Arginine} & 2.0 (2) & 2.2 (2) \\
\text{Valine} & 2.3 (2) & 0.4 (1) \\
\text{Phenylalanine} & 2.9 (3) & 2.0 (2) \\
\text{Alanine} & 0.7 (1) & 0.4 (1) \\
\text{Cystine} & 0.4 (1) & 0 \\
\text{Proline} & 3.0 (3) & 5.0 (5) \\
\text{Leucine} & 0.8 (1) & 0.4 (1) \\
\text{Threonine} & 0.6 (1) & 0 \\
\text{Histidine} & 2.6 (3) & 1.8 (2) \\
\text{Methionine} & 2.0 (2) & 1.4 (2) \\
\text{Tryptophan§} & 2.0 (2) & \text{Not determined} \\
\text{N-Terminal group} & \text{None (tentative)} & \text{Aspartic acid} \\
\end{array}
\]

Molar ratios were determined by the Levy procedure (19).
* The data were taken from the unpublished results of Geschwind and Li.
\dagger The figures in parentheses represent suggested whole integers.
§ The values for these two amino acids were not corrected for hydrolytic destruction.

\( \dagger \) Tryptophan was estimated from ultraviolet light absorption after subtracting tyrosine from the total value.

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components were very close to those of α-MSH and β-MSH, respectively (Fig. 3).

Amino Acid Composition of α-MSH and β-MSH—α-MSH (Fraction HPF-1) prepared by us and β-MSH isolated by Geschwind and Li were hydrolyzed and assayed for amino acids by the Levy procedure (19). Tryptophan was determined by ultraviolet absorption after subtracting tyrosine from the total value (20). Seventeen amino acids were found in α-MSH and their molecular ratios are presented in Table III. The analyses did not give quantitative recovery of amino acids. An estimation made from the alkali consumption during the treatment with 1,2,4-fluorodinitrobenzene accounted for only about 50 per cent of the sample tested. This low yield may be explained in part by the fact that tryptophan, anions, moisture, and ash were not included in the total amino acid percentage. In addition, it is possible that some impurities may have come from the filter paper used.

β-MSH contains thirteen known amino acids (Table III) and probably tyrosine and tryptophan. Aspartic acid was found to be the N-terminal group of β-MSH, whereas a similar sample of α-MSH yielded no N-terminal group when the 1,2,4-fluorodinitrobenzene method was used (21). It is evident from the data in Table III that α- and β-MSH have different amino acid compositions and that they are not the same as the corticotropins from hog and sheep (22–24). The molar ratios for histidine, aspartic acid, and glutamic acid are 3, 1, and 3, respectively, for α-MSH and 2, 4, and 4 for β-MSH. These findings are consistent with the fact that the isoelectric point of α-MSH is basic (pH 11) and that of β-MSH is acidic (pH 5.5).

DISCUSSION

The hormone obtained in the study reported here represents the main MSH component of hog posterior pituitary gland. The activity of the product was approximately 500 times greater on a weight basis than the starting material. However, purified MSH is unstable; it is possible that the potency of the hormone is greater than 1 to 2 × 10¹⁰ units per gm. as reported here. The purified hormone was homogeneous by paper electrophoresis at different pH values and by counter-current distribution. Nevertheless, other criteria of homogeneity remain to be satisfied.

The relation of ACTH to MSH has been controversial as discussed elsewhere (4). ACTH activity was not detected when MSH isolated in this study was tested by the method of ascorbic acid depletion. Also, vasopressin activity was not found. However, less than 0.5 mg. of MSH was used to test for ACTH and vasopressin; it is possible that larger quantities might show some biologic action, even though negligible, similar to those of ACTH and vasopressin. Assay in our laboratory of White's corticotropin
A (23) and Bell’s β-corticotropin (24), which probably represent the main ACTH hormone of hog pituitary gland, showed $1.7 \times 10^6$ MSH units per gm. Thus, purified ACTH has 1 per cent MSH activity either on the basis of contamination or as an intrinsic part of the molecule. The molecular ratios of amino acids in ACTH and MSH are different (22–24). In addition, hog posterior pituitary powder has approximately 10 times as much MSH activity but only one-tenth as much ACTH activity as that from the anterior portion of the gland. According to the distribution of MSH and ACTH in the pituitary gland, the biologic properties of the purified hormones, their amino acid composition, and physical and chemical characteristics, they are distinct substances. MSH was not found to have any intrinsic ACTH activity, but ACTH could have as much as 1 per cent intrinsic MSH activity.

In spite of all the evidence indicating that the two hormones are separate, one remaining source of difficulty is that, in man and animals, elevated blood and urine levels of one hormone seem to be associated with an elevation in the other. Furthermore, when cortisone or hydrocortisone is given to a patient having increased levels of ACTH and MSH, both hormones are reduced to normal (4, 25, 26). It seems as if production of the two hormones is similar and is regulated by like factors. This finding is not incompatible with individuality of the two hormones.

SUMMARY

It was demonstrated that two distinct melanocyte-stimulating hormones (α- and β-MSH) are present in hog pituitary gland. The main active component (α-MSH) was isolated from hog posterior pituitary powder as a fraction homogeneous by counter-current distribution and paper electrophoresis at different pH values. Physical and chemical properties and amino acid composition of α-MSH are described. MSH possesses no detectable ACTH or vasopressin activity.

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ISOLATION OF MELANOCYTE-STIMULATING HORMONE FROM HOG PITUITARY GLAND

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