When aqueous extract of bovine pituitary gland was fractionated by high pressure liquid chromatography (HPLC), the effluent contained a prominent peak of melanotropic activity which did not correspond to α-melanocyte-stimulating hormone (MSH), β-MSH, adrenocorticotropic hormone, or β lipotropin. The novel factor (labeled "post-α-MSH"), responsible for one-third of the melanotropic activity in the gland, was isolated by acetate acid extraction, fractional precipitation with organic solvents, and HPLC. Mobility of post-α-MSH was the same as of α-MSH in zone electrophoresis and paper and thin layer chromatography (TLC), but differed on reverse phase TLC. Amino acid compositions of acid hydrolysates of post-α-MSH and α-MSH were identical. Melanotropic and lipolytic potencies were also the same. Post-α-MSH contained neither glutamine nor carbohydrate. As in α-MSH, both termini were blocked. When post-α-MSH was incubated at pH 9 to 12, it spontaneously converted to α-MSH. HPLC analyses of fragments obtained after enzymatic digestions of both peptides showed that post-α-MSH was a structural variant of α-MSH with N,O-diacetylserine as its NH₂-terminal residue.

Post-α-MSH is responsible for about one-third of the melanotropic activity in aqueous extracts of cattle, rat, guinea pig, or rabbit hypophysis. When the gland is incubated in vitro, >90% of the melanotropic activity released represents post-α-MSH. Physiologic secretion of α-MSH, therefore, may be related to acetylation of the hydroxy group of serine 1.

Peptide IIIF, a previously described melanotropic preparation from choroid plexus, was found by HPLC to contain 1% α-MSH and 99% melanotropically inactive material.

Bovine pituitary gland contains four major melanotropic peptides: α-MSH, β-MSH, ACTH, and β lipotropin (1, 2). Their molecular weights are 1600, 2100, 4500, and 10,000, respectively. All four compounds are synthesized from a single precursor molecule (3). α-MSH, which is acetylated at the NH₂ terminus and amidated at the COOH terminus, is a post-translationally modified derivative of ACTH. We recently noted that, when bovine pituitary extract was fractionated by high pressure liquid chromatography (HPLC), the effluent contained a prominent peak of melanotropic activity which did not correspond to any of these four peptides. A study, therefore, was undertaken to isolate the unknown pituitary melanotropic factor and determine its structure. It was found to be a previously undescribed acetylated form of α-MSH.

Melanotropic activity is also present, although in lesser amounts, in extrahypophyseal regions of the brain (4). Accordingly, HPLC profiles were also determined for the melanotropic activity in certain of these regions.

EXPERIMENTAL PROCEDURES

Materials

Bovine pituitary glands, obtained from PelFreeze, were frozen within 30 min and kept on dry ice until extracted in the laboratory. Rabbit, guinea pig, and rat glands were removed from adult male animals immediately postmortem.

Whole bovine brains, obtained at a local abattoir, were dissected into cerebrum, hypothalamus, pineal gland, and choroid plexus. The same four regions were dissected from fresh rabbit, guinea pig, and rat brains.

Synthetic α- and β-MSH were obtained from Bachem, and oxycellulose-purified bovine ACTH (100 corticotrophic units/mg) was from Sigma. Porcine β-lipotropin was prepared according to Li et al. (2). N- and O-acetylated were from Sigma. Peptide IIIF, a melanotropic factor present in several extrahypophyseal regions of mammalian brain (4), was purified from bovine choroid plexus (4). Oxycellulose was from Eastman. Leucine aminopeptidase, carboxypeptidase A and B, trypsin, and chymotrypsin were from Worthington.

Synthesis of N,O-Diacetylserine

This compound was synthesized by acetylation of O-acetylserine by the method of Folsch et al. (5). The melting point of the crystalline N,O-diacetylserine was 136°C (uncorrected, from literature: 137°C). Chromatographic Analyses

HPLC

HPLC was done on a Waters instrument model 204, with a variable wavelength UV detector according to conditions described in Table I (modified from Ref. 6). The flow rate was 2 ml/min; effluent was collected in 2-ml fractions. Peptides detected by UV absorption were recovered by evaporation and characterized by quantitative amino acid analyses (7), melanotropic (8) and lipolytic (9) bioassays, and behavior in other chromatographic (10) or electrophoretic (10) systems. Experimental details for thin layer chromatography (TLC) (11), reverse phase TLC (12), paper chromatography (10), and high voltage electrophoresis (HVE) (10) are given in Table II. Chymotryptic and tryptic digests of α-MSH and related peptides were prepared by incubating 100 μg of peptide with 1 μg enzyme for 1 h at 37°C in 0.1 M sodium bicarbonate, pH 7.4. α-MSH and related peptides (100 μg) were digested with aminoendopeptidase or carboxypeptidase A plus B (1 μg) for 1 h at 37°C in 0.1 M sodium bicarbonate, pH 7.4. Enzymatic digests were examined by HPLC (6), HVE (1), and paper chromatography (10). Peptides visualized by ninhydrin staining of zone electropherograms and chromatograms were eluted with 1 N acetic acid, dried, and analyzed for amino acid composition.

Sialic acid, hexosamines, glucose, galactose, mannose, and fucose were measured by the method of Rickert and Sweeley (13).
Gas-Liquid Chromatography (GC) Determination of Acetyl Contents of Peptides

Column and Experimental Conditions—The procedure used was based on those of Ward and Coffey (14) and Remsey and Demigne (15). We used a 6-foot glass column (2 mm internal diameter) packed with 5% FFAP (an acid-substituted Carbowax, 20 M, phase) on Gas Chrom Q-80/100 mesh (Applied Science Laboratories). The injector port was at 160°C, the detector was at 190°C, and the column was kept at 120°C. The flow rates were: carrier gas (nitrogen), 55 ml/min; hydrogen, 43 ml/min; and air, 300 ml/min. These conditions were found to be effective for resolution of acetic acid.

Analyses of Standards and Peptides—Known amounts of acetic acid (0.5 to 3.0 µg in 1- to 3-µl aliquots) in 6 N HCl were injected into the column and the area of the peak corresponding to acetic acid was measured to set up a standard curve. Known amounts of N-acetylserine and peptide fragments were hydrolyzed (6 N HCl, 110°C, 16 h) and analyzed under the above conditions; from the area of the acetic acid peak, the acetyl content of the serine derivative or peptide was calculated.

Propionic acid was the internal standard used in these experiments. All samples (standards and peptide hydrolysates) were run in triplicate and their average peak area was determined.

Bioassays

Melanotropic activity was measured on frog skin in vitro (8). In this assay, sections of frog skin are incubated in beakers containing 20 ml of frog Ringer's solution at room temperature with frequent washing until reflectance (measured with a Photovolt model 670 reflectometer) stabilizes. Ten to one hundred units of melanotropic activity then are added to each beaker; each dose being tested in quadruplicate, and 45 min later the skin's reflectance is measured again. The increment in reflectance (ΔR) is linearly related to melanotropic activity over a 10- to 100-unit dose range. Lipolytic activity was examined in vitro on rabbit and rat adipose tissue slices (9) and in vivo in the rabbit (14). Corticotropic activity was tested on the adult rat adrenal gland in vitro (16).

RESULTS

Pituitary Gland

An Unidentified Melanotropic Peak

The elution position of the standards during HPLC is shown in Fig. 1A. With the program used, β-MSH eluted at 5 min, α-MSH eluted at 8 to 10 min, ACTH eluted at 25 min, and β-lipotropin did not appear up to 40 min.

Bovine pituitary glands were homogenized in 0.9% NaCl, pH 7.0 (10 mg/ml wet weight), and the extract was clarified by centrifugation at 30,000 × g for 15 min at 3°C. It contained 9500 units of melanotropic activity/ml. One milliliter of the extract was fractionated on HPLC by system 1 (Table I). The effluent was assayed for melanotropic activity with the result shown in Fig. 1B: 18% of the activity was eluted at 4 to 5 min in the position of β-MSH, 34% at 7 to 8 min in the position of α-MSH, and 34% was eluted at 10 to 12 min. The latter material was labeled "post-α-MSH."

Similar results were obtained with pituitary glands of rat, rabbit, and guinea pig (Fig. 1; Table II).

Isolation of Post-α-MSH and Proof of Homogeneity

The flow sheet is shown in Fig. 2. The final product was obtained in a yield of 0.015 mg/g of bovine hypophysis (wet weight). Homogeneity of the final product was demonstrated by the methods shown in Table III. For comparative purposes, α-MSH was also isolated in homogeneous form by the same scheme (Fig 2; Table III), with an average yield of 0.010 mg/g wet tissue. Purified post-α-MSH and α-MSH both possessed melanotropic activity in the range 0.7 to 1.4 × 10⁻⁴ units/g.

Structure of Post-α-MSH

Relation to α-MSH—The amino acid composition of the 6 N HCl hydrolysate of the purified preparation was identical to that of α-MSH (Table IV). A pronase digest revealed no glutamine. No carbohydrate was detectable on GC analysis of the acid hydrolysate (13). In the series of chromatographic and electrophoretic systems shown in Table III, post-α-MSH behaved differently from α-MSH only in HPLC at pH 4.0 (elution time, 10 to 12 min and 7 to 8 min, respectively) and in reverse phase TLC (Rf 0.45 and 0.38, respectively). In both systems, post-α-MSH exhibited a more hydrophobic behavior than α-MSH.

When purified post-α-MSH was analyzed by HPLC (System 1, Table I), it eluted as one peak at 10 to 12 min. When post-
10104

**Table II**

<table>
<thead>
<tr>
<th>Melanotropic activity</th>
<th>% of extract's activity recovered in each HPLC position</th>
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<tbody>
<tr>
<td></td>
<td>units/mg wet tissue</td>
</tr>
<tr>
<td>Bovine pituitary gland extract</td>
<td>800 ± 10</td>
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<tr>
<td>Bovine hypothalamus extract</td>
<td>110 ± 18</td>
</tr>
<tr>
<td>Bovine choroid plexus extract</td>
<td>90 ± 15</td>
</tr>
<tr>
<td>Bovine cerebral cortex extract</td>
<td>1 ± 0.08</td>
</tr>
<tr>
<td>Bovine pinal gland extract</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>Rat pituitary gland extract</td>
<td>800 ± 190</td>
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<tr>
<td>Rabbit pituitary gland extract</td>
<td>1230 ± 160</td>
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<tr>
<td>Guinea pig pituitary gland extract</td>
<td>680 ± 55</td>
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<td>Rat pituitary gland incubate</td>
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<tr>
<td>Rat pituitary gland incubate, 30 min</td>
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</table>

α-MSH was incubated at 25°C at pH 9 to 12 for 1 to 12 h and then analyzed by HPLC, a variable amount converted to a product which had the same elution position as α-MSH (i.e., 7 to 8 min) (Fig. 3). The conversion product, isolated by HPLC, was indistinguishable from α-MSH in its mobility in all systems shown in Table I, including reverse phase TLC and HPLC, and also in its melanotropic potency. Extent of conversion was dependent on time, temperature, and pH (Table V).

**Digestion with Exopeptidases or Trypsin**—Incubation with leucine aminopeptidase and carboxypeptidase A plus B did not release any free amino acids from either post-α-MSH or α-MSH.

Tryptic digestion of α-MSH (Fig. 4) produced two fragments (as originally reported by Harris in Ref. 1) which were readily separated by HPLC (System 2, Table I) (Fig. 5): T1/α-MSH eluting at 6 min and T2/α-MSH eluting at 4 min. Tryptic digestion of post-α-MSH under the same conditions followed by HPLC gave rise to two fractions with the same amino acid compositions as T1/α-MSH and T2/α-MSH, respectively (Fig. 5 and Table IV). T2/post-α-MSH co-eluted with T2/α-MSH; T1/post-α-MSH, however, eluted later than T1/α-MSH. Thus, T2/post-α-MSH, although its acid hydrolysate possessed the same amino acid composition as the hydrolysate of its α-MSH counterpart (Table IV), showed more hydrophilic behavior. When T1/post-α-MSH was incubated at 25°C, pH 9 to 12, it converted to a product with the same elution position as T1/α-MSH. These data suggested that the structural difference between post-α-MSH and α-MSH was located in the 1 to 8 region.

**Digestion with Chymotrypsin—HVE and paper chromatographic analysis of the chymotryptic digests, with amino acid...**
Amino acid composition of α-MSH, post-α-MSH, and certain tryptic and chymotryptic fragments of each

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>α-MSH*</th>
<th>Post-α-MSH</th>
<th>T1 α-MSH</th>
<th>T2 α-MSH</th>
<th>T3 post-α-MSH</th>
<th>T4 post-α-MSH</th>
<th>C1 α-MSH</th>
<th>C2 post-α-MSH</th>
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<tbody>
<tr>
<td></td>
<td>Theor</td>
<td>Obs</td>
<td>Theor</td>
<td>Obs</td>
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<td>Obs</td>
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<td>Proline</td>
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<td>1.10</td>
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<td>0.92</td>
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<td>Phenylalanine</td>
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<td>0.99</td>
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<td>0.96</td>
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</table>

Fig. 3. HPLC of purified post-α-MSH (System 1, Table I) before and after incubation at pH 12. ΔA (mean ± standard error of four observations in one assay) represents melanotropic activity of the effluent; ΔA, absorbance at 280 nm.

analysis of the eluted bands, was done according to Harris (1). We found for α-MSH and post-α-MSH the five fragments described by that author, viz., residues 1 to 2 (C1), residues 3 to 7 (C3), residues 8 to 13 (C4), residues 8 to 9 (C5), and residues 10 to 13 (C6). No difference in electrophoretic or paper chromatographic mobility could be detected for any of the five fragments of post-α-MSH compared to their counterparts derived from α-MSH.

The chymotryptic fragments of α-MSH were not completely separated by the HPLC program used (System 3, Table I) (Fig. 5). Of the chymotryptic fragments, only the C1 fragment (N-acetyl-Ser-Tyr) eluted as a single peak during HPLC; its elution time was 14 min. This peak will be termed CI/α-MSH.

The other peaks, CPAJCIα-MSH or CPAJCl/α-MSH, contained only serine on amino acid analysis; its elution position was the same as Cl/α-MSH or free tyrosine. The other peak, CPAJCI/α-MSH, contained only serine on amino acid analysis, but it differed in HPLC position from CPAJCl/α-MSH or N-acetylserine (5 min compared to 8 min, respectively).

N-Acetylserine, O-acetylserine, and N,O-diacetylserine eluted from HPLC in System 3 (Table I) at 3, 5, and 8 min, respectively. As stated above, N-acetylserine corresponded in its elution position to CPAJCl/α-MSH or α-MSH. O-Acetylserine did not correspond in elution time to any peak in the chymotryptic digest of α-MSH or post-α-MSH. But N,O-diacetylserine eluted from HPLC in the same position as CPAJCI/α-MSH. These findings suggested the structure N,O-diacetylseryltyrosine for Cl/post-α-MSH.

GC Determination of Acetyl Contents of Peptides

Standardization—A standard curve was prepared to correlate amount of acetic acid injected and area of peak.

Peptide Analyses—The amounts of free acid generated by hydrolysis of 7 to 10 nmol of C1 fragment (acyl-Ser-Tyr) from α-MSH, post-α-MSH, N-acetylserine, and N,O-diacetylserine were measured (Table VI). Only one peak, corresponding to acetic acid, was detected by GC of the acid hydrolysates. The amount of acetic acid recovered from C1 of post-α-MSH was twice that recovered from C1 of α-MSH: 1.6 mol of acetic acid/mol of C1/post-α-MSH versus 0.8 mol/mol of C1/α-MSH. Corresponding recoveries of acetic acid were obtained with hydrolysates of N-acetylserine and N,O-diacetylserine.

We now concluded that post-α-MSH was N,O-diacetylseryltyrosine; α-MSH.

Incubation of Fresh Pituitary Glands

When freshly removed rat pituitary glands were extracted

Table V

<table>
<thead>
<tr>
<th>Temperature</th>
<th>pH</th>
<th>Solution</th>
<th>Duration</th>
<th>% Conversion</th>
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<td>°C</td>
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<td></td>
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<td></td>
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<tr>
<td>37</td>
<td>1.0</td>
<td>0.1 N HCl</td>
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<tr>
<td>37</td>
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<td>0.01 M NH₄ acetate</td>
<td>18 h</td>
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<tr>
<td>37</td>
<td>9.0</td>
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<td>18 h</td>
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<td>37</td>
<td>12.0</td>
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<td>18 h</td>
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<tr>
<td>37</td>
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<td>30 min</td>
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<td>37 ± 5</td>
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<td>5</td>
<td>12.0</td>
<td>0.01 N NaOH</td>
<td>1 h</td>
<td>5 ± 1</td>
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</tbody>
</table>

When freshly removed rat pituitary glands were extracted...

...When freshly removed rat pituitary glands were extracted...
10106

N,O-Diacetylseryl, α-MSH

X = N-acetyl for α-MSH
X = N,O-diacetyl for post-α-MSH

Tryptic digests

Chymotryptic digest, post a-MSH

Fig. 4. Structure of α-MSH and fragments.

Fig. 5. HPLC analysis of tryptic, chymotryptic, and carboxypeptidase digests of α-MSH and post-α-MSH (HPLC Systems 2 and 3 in Table I).

Table VI

Recoveries of acetic acid by GC analysis of hydrolysates of C3 fragments and serine derivatives

| Material hydrolyzed | Theoretical amount of acetic acid | Observed amount of acetic acid | Acetic acid recovered/
|---------------------|-------------------------------|-------------------------------|---------------------|
| C3/α-MSH, 3.1 µg, 10 nmol | 10 nmol | 7.83 nmol | 0.78
| C3/post-α-MSH, 3.5 µg, 10 nmol | 20 nmol | 15.5 nmol | 1.55
| N-acetylseryl, 1.03 µg, 7 nmol | 7 nmol | 5.5 nmol | 0.76
| N,O-Diacetylseryl, 2.0 µg, 21.2 nmol | 21.2 nmol | 16.5 nmol | 1.56

in pH 7.0 aqueous buffer (10 mg/ml), the extract contained about 8000 melanotropic units/ml. HPLC analysis of the extract showed activity to be distributed between the β, α, and post-α elution positions in the ratio 0.7:1.0:1.1 (Table II). When the fresh gland was incubated in Krebs-Ringer bicarbonate solution containing 1 mg/ml of glucose at 37°C under 95% O2, 5% CO2, activity was released at a rate of 75 units of melanotropic activity/mg of wet tissue/h (i.e. 10% of the activity in the gland was released/h). When the medium was analyzed by HPLC, the ratio of activities in the β/α/post-α elution positions was 0.3:1.0:1.5 (Table II).

Generally similar results were obtained on incubating fresh guineas pig or rabbit glands.

Comparison of Potencies of α-MSH and Post-α-MSH

The results are illustrated in the form of dose-response curves in Fig. 6. The two preparations possessed the same potencies in the melanotropic assay (about $1 \times 10^{-8}$ units/g) and also in both in vitro and in vivo lipolytic assays. Neither was detectably active in stimulating steroidogenesis by the adult rat adrenal gland (<1 $\times 10^{-9}$ as potent as ACTH).

Other Regions

Aqueous extracts (pH 7.0, 0.9% NaCl) were made of bovine hypothalamus, cerebrum, pineal gland, and choroid plexus (10 ml/mg). One milliliter of extract was analyzed by HPLC. The patterns of melanotropic activity are shown in Fig. 1 and Table II. Similar results were obtained for cattle and rabbit extracts. In the hypothalamus, pineal gland, and choroid
the scheme in Fig. 2. 45% of the added MSH activity was synthetically added to a 10 times greater quantity of synthetic α-MSH than naturally present was added, the homogenate was centrifuged, and the extract was analyzed by HPLC as in Fig. 1. Essentially all the added MSH activity was recovered in the α position. Similarly, a 10 times greater quantity of synthetic α-MSH than naturally present was added, the homogenate was centrifuged, and the extract was analyzed by HPLC as in Fig. 1. Essentially all the added MSH activity was recovered in the α position. Similarly, synthetic α-MSH was added to a 10× acetic acid homogenate of bovine cerebrum and the tissue was processed according to the scheme in Fig. 2. 45% of the added MSH activity was recovered in the α-MSH fraction; no melanotropic activity was recovered in the post-α-MSH fraction.

**DISCUSSION**

These experiments confirm the presence of α- and β-MSH in subhuman mammalian pituitary glands. The data identify a novel factor: N-O-diacetylseryl, α-MSH or post-α-MSH. This novel factor accounts for 27 to 40% of melanotropic activity in the pituitary gland of all 4 species studied.

The newly isolated diacetyl form of α-MSH could be distinguished from α-MSH only on the liquid chromatography column (μBondapak C-18) using 25% CH_3CN in 0.01 M ammonium formate as the eluant and on reversed phase TLC on Whatman KCl_2 thin layer plates with 30% CH_3CN in 0.01 M ammonium formate at pH 4.0 (cf. Table III). This explains why this form of α-MSH was not identified earlier in work on the hormone (1) since reverse phase TLC and HPLC were not available. Furthermore, Harris identified N-acetylseryl, essentially on the basis of qualitative paper chromatographic analysis (1); no quantitative estimate of acetic acid in the acid hydrolysate was made.

Diacetyl α-MSH is not an artifact since it is present in pH 7 aqueous extracts of fresh pituitary glands. Moreover, when synthetic, monoacetyl α-MSH was added to pituitary gland or cerebrum prior to extraction or purification of diacetyl α-MSH, no detectable amount of the former was converted to the latter.

The spontaneous conversion of post-α-MSH to α-MSH, of T_1 post-α-MSH to T_1/α-MSH, and of C_1/post-α-MSH to C_1/α-MSH, is consistent with the proposed structure. O-Acetyl (ester) groups are relatively easier to cleave than N-acetyl (amide) groups by base-catalyzed nucleophilic substitution (18). Experimental conditions required to convert C_1/post-α-MSH to C_1/α-MSH are milder than those required for converting post-α-MSH to α-MSH. This can be at least partially explained in terms of the reduced steric hindrance offered to the nucleophilic anion approaching the acetylated dipeptide.

While α-MSH and N,O-diacetyl-Ser, α-MSH are present in the fresh gland in roughly equal amounts, only the latter is found in the incubation medium. This observation suggests that α-MSH may need to be acetylated before it is released from the gland. Indeed, since the wet gland contains 30 to 50% extracellular fluid (estimated from Cl^- concentration), some of the diacetyl α-MSH in the gland may already be extracellular.

Extrahypophyseal areas show two patterns: the circumventricular regions resemble the hypophysis in possessing approximately equal proportions of melanotropic activity in β/α/post-α positions averaged 0.31/0.65.7. In cerebrum, however, α predominated, with the average ratios being 0.01/0.99/0.01.

**Acknowledgments**—We thank Drs. D. P. Groth and R. Shapira for helpful discussions.
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