former yields 3.6 N-terminal valyl residues and the latter 3.2 N-terminal valyl residues per molecule of hemoglobin.

Conclusions

Since our results differ from those of previous investigators, it seems important to summarize the points of similarity and difference. The following similarities should be noted: (1) the equivalence of DNP-globin to hemoglobin determined in these experiments from the amino acid content is in excellent agreement with Sanger's value determined from the amide nitrogen content, and (2) the nature of the DNP-protein hydrolyzed is the same as that of other investigators. The following differences are apparent: (1) our correction factor for the destruction of DNP-valine during acid hydrolysis is much less than that of other investigators, and (2) unhydrolyzed DNP-peptide that remains at the end of the hydrolysis has been quantitatively estimated in the present work.

The low destruction of DNP-valine that we have observed in our analyses has been discussed in a preceding section. Experiments to determine the cause of the difference cannot be devised because details about the conditions of hydrolysis are lacking in the other papers. Throughout the present investigation great stress has been laid upon the exact determination of the loss of DNP-valine during hydrolysis. To this end, not only DNP-valine but DNP-val-gly and DNP-val-leu have been hydrolyzed both in the presence and in the absence of DNP-globin. The consistent results reported in Table II lead us to conclude that confidence may be placed in the correction factor which has been determined. By use of this correction factor it has been shown that 0.1000 g. of DNP-globin, as prepared from purified hemoglobin and hydrolyzed according to the methods described in the present paper, contains 4.04 ± 0.15 μmole of DNP-valine.

If the above data and the commonly accepted value of approximately 67,000 for the molecular weight of human hemoglobin are then used to calculate the number of end groups, the non-integral value 3.6 ± 0.1 end groups is obtained. For the integral value of 4, a molecular weight of about 76,000 is required; for the integral value 3, about 57,000. The literature provides no evidence supporting a molecular weight of 76,000, but the work of Field and O'Brien[43] and of Srinivasan and Vinograd[44] suggests that the molecular weight may be about 60,000. On the other hand, if the non-integral value of 3.6 end groups per molecule is correct, it implies that preparations of hemoglobin that have been considered to be homogeneous are indeed heterogeneous. Although heterogeneities in normal adult human hemoglobin have been reported by Kunkel and Wallenius,[10] Morrison and Cook,[45] and Prins and Huisman,[46] it should be noted that uncrystallized hemoglobin was used in the experiments of these investigators.

Despite the immense amount of work that has been done on hemoglobin, it is apparent that uncertainties still exist about both the molecular weight and the homogeneity of this protein. Consequently, a final decision as to the exact number of N-terminal residues must be kept in abeyance until both the molecular weight of hemoglobin and the homogeneity of the preparations are established with more certainty.

Acknowledgments.—We wish to express our appreciation to Dr. M. Murayama for supplying several samples of hemoglobin and for carrying out the electrophoretic tests for homogeneity, and to Drs. R. Srinivasan and J. Vinograd for making available their unpublished work on the molecular weight of human hemoglobin. This investigation was supported in part by a research grant (RG-4276) from the National Institutes of Health, Public Health Service.

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CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY AND THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Isolation and Characterization of a Melanocyte-stimulating Hormone (β-MSH) from Hog Pituitary Glands

BY IRVING I. GESCHWIND AND CHOH HAO LI

RECEIVED AUGUST 31, 1956

The isolation of a melanocyte-stimulating hormone (β-MSH) from porcine posterior pituitary powder has been described. The active material was extracted with warm glacial acetic acid and further purified by acetone and ether precipitations. It was then adsorbed on oxycellulose, eluted and further purified by zone electrophoresis on starch. Finally, it was submitted to countercurrent distribution. Amino acid analyses of the isolated product indicated the following composition: $Asp_2Glu_2Ser_1Gly_2Pro_3Met_1Phe_1Tyr_1Lys_2His_1Arg_1Try_1$. The molecular weight, 2177, calculated from these data, is shown to be the true molecular weight. The isoelectric point, determined in buffers of 0.1 ionic strength, has been found to be at *p*H 5.8. A comparison has been made between the isolated peptide and α- and β-MSH reported previously by other workers.

The presence of a melanocyte-stimulating hormone (MSH, intermedin) in the *pars intermedia* of the pituitary was first demonstrated by Zondek and Krohn in 1932.[1] Thereafter, few intensive investigations designed to isolate the pure hormone

(1) B. Zondek and H. Krohn, *Klin. Wochschr.*, **11**, 405 (1932).

were carried out until about 5 years ago when interest in this hormone was renewed. The results of these earlier efforts have been summarized by Waring and Landgrebe.[2] In the course of the research leading to the isolation of the various corticotropins, it became apparent that crude corticotropic preparations possessed greater melanocyte-stimulating activity than did any of the actual MSH preparations then available. However, the bulk of the melanocyte-stimulating activity in the corticotropins could be separated from the adrenal-stimulating activity, although some of the former activity was always found to be associated with the latter.[3] With the isolation of pure corticotropins during the past few years, it has been possible to demonstrate that the melanocyte-stimulating activity is an intrinsic activity of these peptide hormones.[4]

In 1955, Lerner and Lee[5] reported the isolation from porcine pituitary glands of a pure melanocyte-stimulating hormone. Subsequently, Benfey and Purvis[6] and Porath, Roos, Landgrebe and Mitchell[7] reported the purification of a melanocyte-stimulating peptide from porcine glands which differed from the preparation reported by Lerner and Lee. It was suggested by Lee and Lerner[8] that their preparation be termed α-MSH and that the peptide hormone reported by the other investigators be designated β-MSH. The present communication describes the isolation of a pure β-MSH from porcine pituitary glands and reports the partial physicochemical characterization of this peptide. The accompanying paper presents the structure of β-MSH.[9] A preliminary report of these data has appeared recently.[10]

Isolation Procedure

Crude MSH Concentrate.—The preparation of the crude MSH concentrate follows closely, with a few slight modifications, the procedure introduced by Astwood and his colleagues.[11]

Acetone-dried porcine posterior pituitary powder in 200-g. lots with an average activity of 2×10^4 u./mg.[12] was wetted with 500 ml. of acetone and then was extracted with 2 l. of glacial acetic acid at 50° for 20 minutes. The mixture was centrifuged, and the precipitate was re-extracted with 400 ml. of acetic acid, followed by 400 ml. of acetone. To the combined extracts 20 ml. of saturated aqueous NaCl was added, followed by a half volume of acetone. The solution was stirred and then allowed to stand for 1 hr. in an ice-bath. After centrifugation, one volume of diethyl ether was added to the clear supernatant fluid; the ether solution was stirred and then returned to the ice-bath for 2 hr. The clear supernatant fluid was siphoned off and the precipitate was washed extensively with acetone. The washed precipitate was transferred to a desiccator and dried over NaOH. The average yield was 44 g. of material with an average activity of 5×10^4 u./mg.

The dried ether precipitate was dissolved with stirring in 1500 ml. of 0.1 *N* acetic acid; to the clear solution (any insoluble material being removed by centrifugation) 6 g. of oxycellulose (10–12% COOH) was added, and the resulting suspension was stirred overnight. The suspension was then centrifuged, and the oxycellulose thus obtained was washed twice with 0.1 *N* acetic acid and once with water. The adsorbed MSH activity was eluted from the washed oxycellulose with 150 ml. of 0.1 *N* HCl for 1 hr. A clear extract was obtained by centrifugation. The precipitate was re-extracted for 30 minutes with 100 ml. of 0.1 *N* HCl. The combined extracts were deacidified with methyldioctylamine[13] and the deacidified solution was lyophilized. The lyophilized powder (average yield, 600 mg.) was then dissolved in 30 ml. of water and the *p*H was adjusted to 6.5–7.0 with NH_4OH. The precipitate that formed was removed by centrifugation, and the supernatant fluid was lyophilized. The average activity of the lyophilized product was 2×10^6 u./mg.; the average yield of this crude MSH concentrate was 500 mg.

Purification by Zone Electrophoresis.—Further purification of the crude MSH concentrate was achieved by means of zone electrophoresis on starch.[14] The experimental details followed the previously published procedure.[15] For each experiment, two 60-cm. troughs, each containing 150 mg. of the crude MSH concentrate, were used simultaneously with a single pair of electrode-buffer vessels. The material was added to the troughs at a point 10 cm. from the anode end, and the separation was carried out in a 0.1 *M* pyridine-acetate buffer of *p*H 4.9 at 230 volts for 48 hr. at 40°. The contents of the troughs were cut into 1-cm. segments and each segment was eluted 3 times with 4 ml. of water each time. An aliquot of the extract (0.5 ml.) was then analyzed for protein concentration by means of the Folin biuret reagent.[16] A typical pattern is shown in Fig. 1. The major portion of the MSH activity is concentrated in segments 18–23. Extracts of these segments were pooled, filtered and lyophilized. The average yield was 60 mg. (45% peptide, 55% starch); the average activity was 2×10^7 u./mg. (peptide basis).

In order to remove small amounts of contaminants still present (as indicated by the very high baseline, Fig. 1) and to concentrate the material in proportion to the starch prior to countercurrent distribution, re-runs were made with 100 mg. of material that had already been submitted once to zone electrophoresis. The pattern obtained from such a re-run is shown in Fig. 2. Because of the concentration of material in a single large, narrow peak, the dried product contained only about 25% starch.

Purification by Countercurrent Distribution.—The purified material (200–400 mg.) obtained by starch electrophoresis was submitted to countercurrent distribution in the system 2-butanol-aqueous 0.5% trichloroacetic acid. An automatic all-glass apparatus[17] consisting of 240 tubes was employed; all runs were carried out at 20°.

In a typical experiment, after the apparatus was filled with lower phase (5 ml. per tube), the device for automatic addition of upper phase was put into operation. After a forerun of 20 transfers had been completed, the lower phase of the first tube was then removed and to it the entire

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(12) The unit of activity was that defined by K. Shizume, A. B. Lerner and T. B. Fitzpatrick (*Endocrinology*, **54**, 553 (1954)), whose assay method was employed. One I.U. is equal to approximately 10^4 of these units.

(13) D. E. Hughes and D. H. Williamson, *Biochem. J.*, **48**, 487 (1951).

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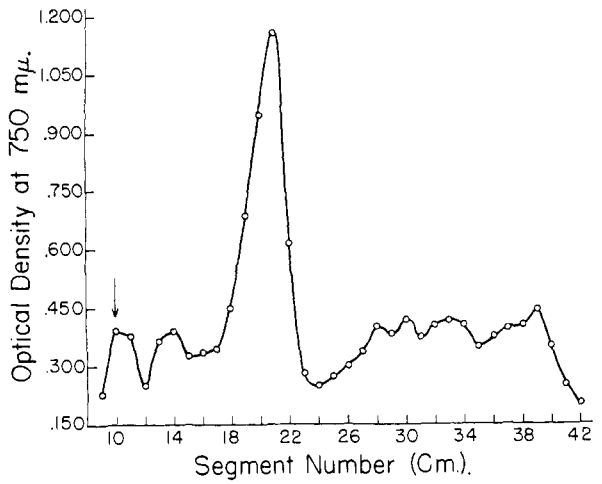

Fig. 1.—Pattern obtained from zone electrophoresis of crude MSH concentrate on starch; 60 cm. trough, 0.1 *M* pyridine–acetic acid buffer at *p*H 4.9; 230 volts, 48 hr. The melanocyte-stimulating activity is concentrated in segments 18–23.

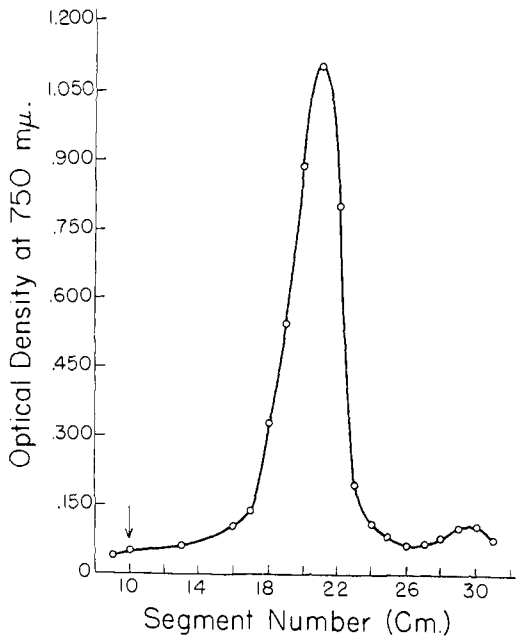

Fig. 2.—Electrophoretic pattern obtained with material that had previously been purified by zone electrophoresis on starch. Conditions the same as in Fig. 1.

amount of material (305 mg. of peptide) was added. Approximately the first 10 transfers required a long settling time, but thereafter this time decreased until a single cycle could be completed in from 4.5 to 5 minutes. After 240 transfers, the lower phases of every fourth tube from tubes 0 to 160 and the upper phase of every fourth tube from 161 to 239 was removed, and the optical densities at 277 mμ were determined in a Beckman model DU spectrophotometer. Most of the material was present as a large peak with a *K* value of 0.7 and as a smaller adjoining peak with a *K* value of 0.4. Small quantities of other materials, present at each end of the apparatus, were removed, and fresh upper and lower phases were added. The machine was then set for cycling and allowed to proceed for 1100 transfers. The main peaks were located by sampling lower phases from every fourth tube, and the contents of all tubes which a peak comprised were analyzed.

The distribution obtained is shown in the upper part of Fig. 3. There are present a main skewed peak with a *K* value of 0.62, a very small peak whose *K* equals 0.45 and a small peak whose *K* equals 0.35. The contents of tubes 376–425 (Pool I) and of tubes 426–456 (Pool II), which make up the main peak, were separately pooled, as were tubes 266–314 which make up the peak with the *K* value of 0.35[18]; each batch of pooled material was concentrated in a

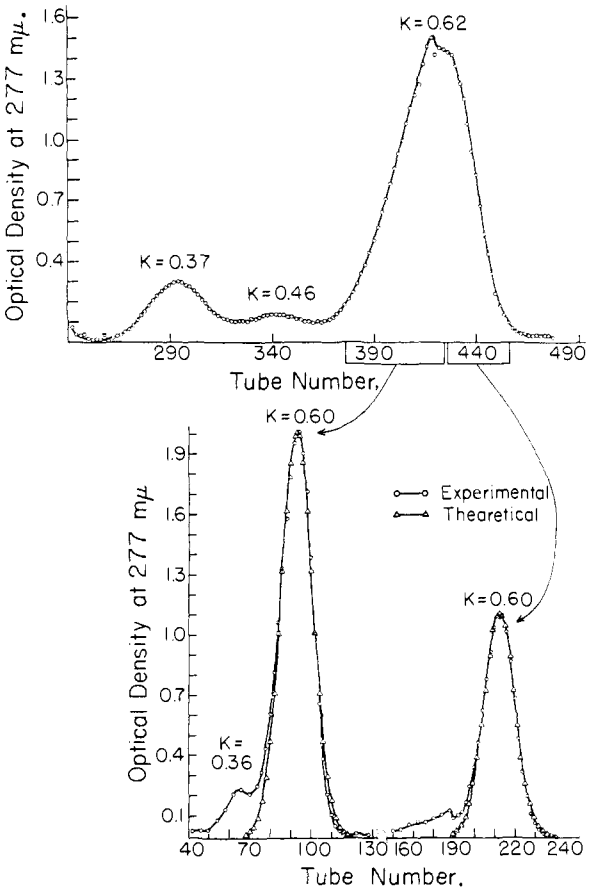

Fig. 3.—Upper, countercurrent distribution (1100 transfers) of material obtained from zone electrophoresis on starch. System, 2-butanol–0.5% aqueous trichloroacetic acid. Lower, countercurrent distribution (248 transfers) of the two halves of the main peak obtained above (upper, Fig. 3). The material found in tubes 376–425 after the first distribution was placed in tube 0 and that found in tubes 426–456 was placed in tube 120. System, 2-butanol–0.5% aqueous trichloroacetic acid.

rotary evaporator and was subsequently lyophilized. The distribution apparatus was then filled with fresh lower and upper phase. The material from Pool I, representing the trailing half of the main peak, was dissolved in the lower phase of the first tube of the upper train, and that from Pool II, making up the advancing half of the main peak, was dissolved in the lower phase of the first tube (tube 120) of the lower train. The machine was set for recycling and allowed to proceed for 248 transfers. Analysis of the lower phases of all tubes revealed the distribution shown in the lower half of Fig. 3. Both peaks have identical partition coefficients ($K = 0.60$), and each is slightly skewed, being steeper than theoretical on the advancing edge and broader than theoretical on the trailing. This is the most evident in the larger peak, which contains about twice as much material as the smaller. The material representing each of the two peaks was individually pooled, concentrated and lyophilized. From the large peak 127 mg. (13.3% N; trichloroacetate)

(18) For the nature of this component, see the Discussion.

and from the smaller 71 mg. (13.5% N; trichloroacetate) were obtained. From 1 kg. of fresh posterior lobes of porcine pituitary glands, approximately 45 mg. of β-MSH could be isolated. The activity was 0.5×10^7 u./mg. The β-MSH isolated by the above procedure has proven to be homogeneous by the criteria discussed below and also by end-group analyses and by structural determinations which will be described in the accompanying paper.[9]

Characterization

Amino Acid Analysis.—Quantitative amino acid analyses were performed on both Pools I and II by the method of Levy.[19] Samples from Pool II, 2 to 3 mg. each, were hydrolyzed at 110° in 5.7 *N* HCl for 24 and 48 hr., while samples from Pool I were digested for the 24-hr. period only. The hydrolysates were dinitrophenylated at *p*H 9 in an autotitrator,[20] and the dinitrophenylated amino acids were then submitted to two-dimensional chromatography on paper. From each sample triplicate chromatograms were prepared. The yellow spots were excised, eluted and the optical densities determined. In this manner the molar ratios for all the amino acids, with the exception of tryptophan, were determined. Tyrosine and tryptophan were determined independently by a spectrophotometric method.[21] Amide determinations made on both unhydrolyzed[22] and partially hydrolyzed[23] preparations gave no indication of the presence of amide ammonia in any of the samples of β-MSH used. The results of all analyses, presented in Table I, suggest the following empirical formula for β-MSH

$$Asp_2Glu_2Ser_1Gly_2Pro_3Met_1Phe_1Tyr_1Lys_2His_1Arg_1Try_1$$

TABLE I

MOLAR RATIOS OF AMINO ACIDS IN β-MSH

	Fractions of β-MSH			Residues to the nearest integer
	Pool I[a]	Pool II[a]		
Amino acid	24 hr.[b]	24 hr.[b]	48 hr.[b]	
Aspartic acid	1.9	1.9	2.1	2
Glutamic acid	2.1	2.1	1.9	2
Serine	0.9	1.0	0.9	1
Glycine	1.9	1.9	2.0	2
Proline	3.1	3.1	3.2	3
Methionine	0.6	0.8	0.7	1
Phenylalanine	1.1	1.0	1.1	1
Tyrosine	0.9(0.9)[c]	0.5(1.0)[c]	0.9	1
Lysine	1.9	1.7	1.9	2
Histidine	1.0	1.0	1.0	1
Arginine	0.9	1.0	1.0	1
Tryptophan	1.1[c]		1.0[c]	1
Amide NH_3	0.0[d]		0.0[e]	..
			Total	18

[a] See text and Fig. 3. [b] Duration of hydrolysis. [c] Determined by spectrophotometric method.[21] [d] Determined on unhydrolyzed sample.[22] [e] Determined on partially hydrolyzed sample.[23]

The minimum molecular weight calculated from such a formula is 2177. From evidence reported below and in the accompanying paper it will be seen that this is also the true molecular weight. The theoretical nitrogen content is 16.7%.

Sedimentation Behavior.—Two different samples of β-MSH trichloroacetate have been submitted to ultracentrifugation in a Spinco ultracentrifuge at 59,780 r.p.m., with a synthetic boundary cell.[24] For the first sample, centrifuged in a buffer of *p*H 2.2 at a concentration of 0.35%, the sedimentation constant was calculated as $S_{20} = 0.50 \times 10^{-13}$ sec. The second sample was centrifuged at a concentration of 1.4% in a borate buffer of *p*H 9.9; the calculated value for the sedimentation constant of this sample, $S_{20} = 0.48 \times 10^{-13}$ sec., is in excellent agreement with the value found at the much lower peptide concentration and *p*H. In each case only a single sedimenting boundary was observed.

Determination of Isoelectric Point.—Electrophoretic mobilities of β-MSH on starch as a function of *p*H were determined by procedures previously described.[25] Acetate, cacodylate and veronal buffers of 0.1 ionic strength were used, and all runs were carried out at 4° with 1 to 2 mg. samples for 24 hr. at a potential gradient of 5 volts per cm. The location of the hormone peptide was determined as described above; at each *p*H only a single component was found. The mobilities were calculated and then corrected for electroösmosis as described by Raacke and Li.[25] As shown in Fig. 4, from a plot of the corrected mobilities *versus* the *p*H in the trough, a value of *p*H 5.8 can be interpolated for the isoelectric point. This value is in excellent agreement with the calculated[22,26] value for the isoionic point (*p*H 5.9), based upon the amino acid composition of the peptide hormone.

Discussion

All the recent methods that have been employed for the purification of MSH made use of the glacial acetic acid extraction technique, orginally introduced by Kamm, *et al.*,[27] for the fractionation of posterior pituitary hormones and later successfully adapted[11a] for the purification of the corticotropins. The use of oxycellulose as a selective adsorbent for MSH also derives from techniques originally introduced for the purification of the corticotropins.[11b] In the application of these methods to the present study, no difficulties were encountered; and with any batch of posterior pituitary powder from a given commercial source, starting with as little as 10 g. or as much as 400 g., both weight and activity yields are reproducible. Differences have been noted, however, in batches of powder obtained from different commercial sources.

Posterior pituitary powders used early in the present work gave ether precipitates which were more than 98% soluble in 0.1 *N* acetic acid. After it had been eluted from oxycellulose, about one-third of this material was insoluble at *p*H 6.5. When the supernatant fluid at this *p*H was submitted to zone electrophoresis, the resulting patterns indicated the presence of 3 additional peaks in concentrations equal to that of the MSH peak, in addition to a large amount of material that remained at the origin. Pituitary powders, obtained from another commercial source, that have been employed more recently have given ether precipitates which are less than 25% soluble in 0.1 *N* acetic acid. In this case, however, over 80% of the material eluted from oxycellulose is soluble at *p*H 6.5. The electrophoretic distribution pattern illustrated in Fig. 1 was obtained with this latter material.

In order to minimize the amount of salt[28] that would be introduced in raising the *p*H of the oxycellulose eluate to 6.5, a deacidification step was introduced into the procedure. An anion-exchange

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(20) C. F. Jacobsen and J. Léonis, *Compt. rend. trav. Lab. Carlsberg, Série chim.*, **27**, 333 (1951).

(21) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

(22) A. L. Levy, I. I. Geschwind and C. H. Li, *J. Biol. Chem.*, **213**, 187 (1955).

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(26) A. D. McLaren and C. Lewis, *J. Polymer Sci.*, **5**, 379 (1950).

(27) O. Kamm, T. B. Aldrich, I. W. Grote, L. W. Rowe and E. P. Bugbee, THIS JOURNAL, **50**, 573 (1928).

(28) For a similar reason, zone electrophoresis was carried out in a pyridine-acetic acid buffer, as the components of this buffer are completely removable by lyophilization.

resin (Amberlite IR-4B) in the hydroxide form was originally employed for the deacidification. It soon became evident, however, that only those materials were being recovered which had the greatest cathodic mobility during electrophoresis, whereas the recoveries of melanocyte-stimulating hormone were poor. The adoption of a procedure using methyldioctylamine as the deacidifying agent[13] permitted full recovery of all materials.

When the *p*H of the deacidified solutions was adjusted over a range of from 3 to 11, it was found that only in the region of *p*H 6.5–7 could any sizable amount of comparatively inactive material be removed, a finding in agreement with the data of Landgrebe and Mitchell.[29] The precipitate obtained in this *p*H region is rich in corticotropic activity.

A concentration of corticotropic activity is also effected during zone electrophoresis; the material possessing this activity can be found in the region between segments 28 and 33 (Fig. 1). This area, and the area comprising segments 36–41 (in which we believe oxytocic activity to be concentrated), were both larger when the earlier batches of posterior pituitary powder were used (*vide supra*). Under no condition of electrophoresis, with either the duration of the run or the *p*H at which migration occurred, as variables, could any material be observed with a mobility suggesting an isoelectric point between *p*H 10.5 and *p*H 11, that found for α-MSH by Lerner and Lee.[5]

As countercurrent distribution studies indicate, material which has been twice subjected to electrophoresis is highly purified. Nevertheless, such material is contaminated with a component(s) that gives rise after acid hydrolysis to alanine, valine and leucine, among other amino acids. Of these 3 amino acids, none of which occurs in β-MSH (see Table I), alanine and valine were present in the purified MSH obtained by Porath, *et al.*,[7] and all 3 together with cystine were found as contaminants in the preparation of Benfey and Purvis.[6] Such contaminating material has been successfully removed in these investigations only by employing a large number of transfers in countercurrent distribution.

After such multiple partitions, however, an entirely satisfactory pattern of distribution has not been obtained. As shown in Fig. 3, a skewness has been observed which appears to be, to some extent, concentration-dependent. Furthermore, it appears that a gradual transformation of the material takes place, evidenced by a change in the distribution coefficient, K, from 0.6 to 0.35. The pattern in the lower half of Fig. 3 gives some indication of this transformation. With still other samples of MSH, the gradual growth of a shoulder on the trailing edge of the main peak has been observed during a run. In one particular instance, when material that had originally partitioned with a K value of 0.62 was redistributed in the same solvent system, it was entirely transformed into a substance with a K value of 0.35. This material, and the material isolated as Pool III from the run

(29) F. W. Landgrebe and G. M. Mitchell, *Quart. J. Exper. Physiol.*, **39**, 11 (1954).

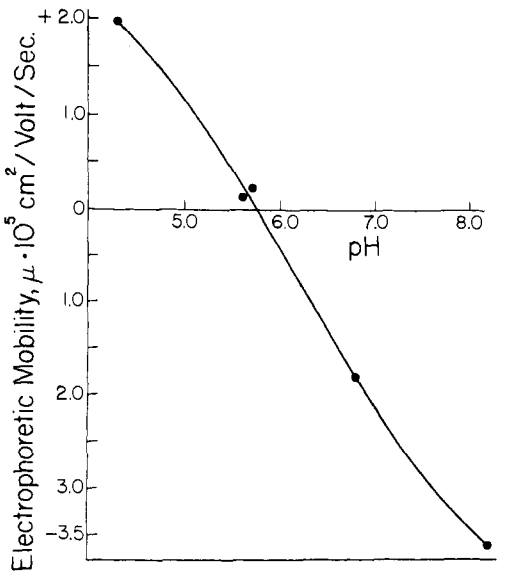

Fig. 4.—Electrophoretic mobility of β-MSH on starch as a function of *p*H. The mobilities have been corrected for electroösmosis.

given in detail in the Experimental section, have single N- and C-terminal amino acids, which are the same as those found for Pools I and II (see accompanying paper); and furthermore, their amino acid composition is identical with that presented in Table I, with the exception of small amounts of leucine, alanine and valine contaminants in Pool III. Aside from their partition ratios, the major difference between these materials with K values of 0.35 and those with K values of 0.6 is that the former, when isolated, possess a very low nitrogen content. Pool III, for example, contained 9.3% nitrogen. When corrected for differing nitrogen content, Pool III is only about one-tenth as active as Pools I and II. Moreover, when the activities of Pools I and II are compared with the material from which they were derived (see Experimental section), it becomes apparent that a gradual transformation leading to inactivation has occurred in the course of the distribution. It has not been possible in practice to attempt a large number of distributions in a cold room, where, it might be hoped, the rate of such inactivation or transformation would be decreased.

With respect to the amino acid analyses, it may be noted that the common amino acids cystine, threonine, isoleucine, leucine, valine and alanine are all absent from β-MSH. With the exception of isoleucine, all are present in α-MSH.[5,8]

The quantitative data obtained from the amino acid analyses reported herein are substantially in agreement with that reported by Benfey and Purvis,[6] if the molar ratios of these authors are divided by two.[30] Then the only differences encountered are their lower values for glycine and proline, their higher value for amide ammonia and, as discussed

(30) Similarly, the amino acid analysis reported by Lee and Lerner[8] for a β-MSH prepared by us should also be divided by two to bring the results into line with the analysis published in this paper. This preparation had not previously been submitted to countercurrent distribution.

above, the small amounts of contaminating amino acids apparently present in their preparation. A similar problem with contaminants is evident in the data of Porath, *et al.*[7]; moreover, methionine is absent from the qualitative analysis presented by these authors, at the same time that they report that no sulfur-containing amino acids had been found to be present. Also, the analysis of 24- and 48-hr. hydrolysates presented in Table I of this paper gives no evidence in support of the claim of these authors that certain peptide bonds in β-MSH are particularly resistant to acid hydrolysis.[7]

The minimum molecular weight of β-MSH calculated on the basis of the amino acid content is 2177, a value which, as it has been pointed out above, represents the actual molecular weight of the hormone peptide. A molecular weight of 2900 for β-MSH has been determined independently by equilibrium centrifugation.[31]

Another value that may be computed from the amino acid composition is the isoionic point of β-MSH; as pointed out earlier, the calculated value for the isoionic point, *p*H 5.9, is almost identical with the experimental value for the isoelectric point, *p*H 5.8, determined in buffers of 0.1 ionic strength. A value of *p*H 5.2 was reported by Porath and co-workers[7] for the isoelectric point of their MSH preparation in 0.05 M phosphate buffers. Both of these values differ considerably from that reported for the isoelectric point of α-MSH,[5] which is between *p*H 10.5 and *p*H 11.

In view of all the foregoing comparative data, there would appear to be little doubt that the MSH peptide purified by Benfey and Purvis and by Porath, *et al.*, is identical with the β-MSH whose isolation has been described in this paper, just as there can be equally little doubt that α-MSH of Lerner and Lee[5] differs considerably. It is of interest that none of the workers, including ourselves, who have attempted to purify β-MSH have reported any evidence for the existence of α-MSH. Although we have specifically sought it in the course of the present work, we have failed to detect melanocyte-stimulating activity in any peptide as basic as α-MSH. At present, there seems to be no obvious explanation for this discrepancy.

Finally, a few words about the assay methods are in order. All of our earlier assays were performed on hypophysectomized *Rana pipiens* and were quantitated by means of the change in the melanophore index[32] as detected in the web. Subsequently, the assay was made more objective by employing the *in vitro* frog skin method.[12] Excellent agreement has been found between assay results obtained with the two methods. Furthermore, the activities reported above are in good agreement with those reported by other workers for β-MSH. Recently Eakin[33] reported that some of our crude MSH concentrate, containing no more than about 10% β-MSH, stimulated the melanocytes of the albino-hypophysectomized tadpole of *Hyla regilla*, when 8×10^{-6} μg. was injected. Hence, this latter method of assay can detect approximately 10^{-6} μg. of β-MSH.

Acknowledgments.—The authors wish to acknowledge the able technical assistance of Miss Winifred Lilly and Mr. David Chung. This work was supported in part by grants from the National Institutes of Health of the United States Public Health Service (Grant No. G2907) and the Albert and Mary Lasker Foundation.

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The Structure of the β-Melanocyte-stimulating Hormone

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RECEIVED AUGUST 31, 1956

By means of chemical and enzymatic degradations, the structure of β-MSH has been shown to be: H-Asp.Glu.Gly.-Pro.Tyr.Lys.Met.Glu.His.Phe.Arg.Try.Gly.Ser.Pro.Pro.Lys.Asp-OH. It has been shown previously that the sequence Met.Glu.His.Phe.Arg.Try.Gly also exists in the corticotropins. The implications of these findings have been discussed.

In the preceding paper[1] the isolation of the β-melanocyte-stimulating hormone (MSH) from porcine pituitary glands has been described and its characterization presented. The hormone has been found to be an octadecapeptide with the following composition: $Asp_2Glu_2Ser_1Gly_2Pro_3Met_1Phe_1Tyr_1Lys_2His_1Arg_1Try_1$. In view of the well-known specificities of trypsin and chymotrypsin, the presence of only 3 residues of lysine and arginine together and of 3 aromatic residues suggested that digestion with either of these enzymes should result in a small number of cleavage products whose structural sequence should be relatively easy to determine. Furthermore, since 7 amino acids are present as single residues in the hormone, the

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